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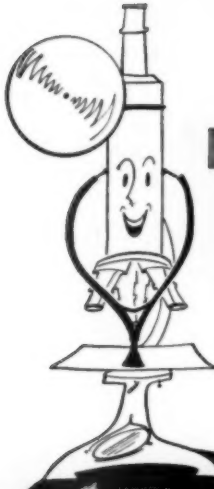

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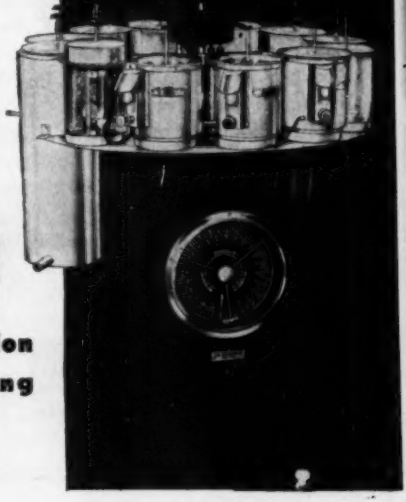

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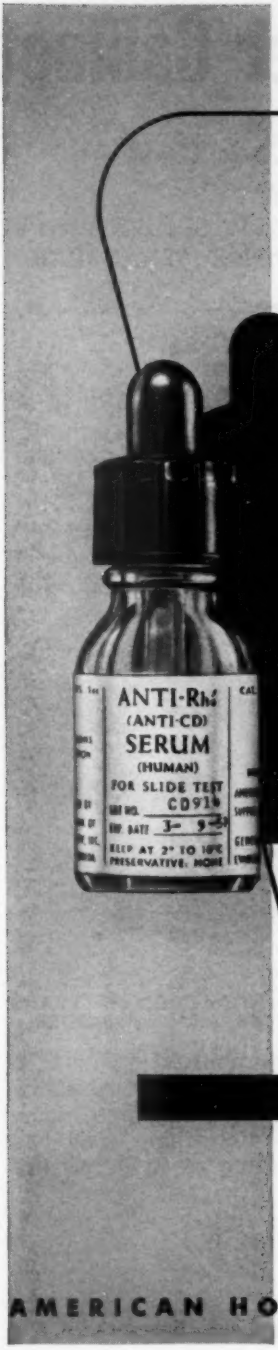

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ARCHIVES OF PATHOLOGY

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HYPERTENSIVE DISEASE PRODUCED BY DESOXYCORTICOSTERONE ACETATE IN PARABIOTIC RATS

C. E. HALL, Ph.D.
AND
O. HALL, M.Sc.
GALVESTON, TEXAS

DESOXYCORTICOSTERONE acetate produces arterial hypertension in a variety of animal species, including man.[1] In the rat such hypertension results in widespread vascular damage, typified by nephrosclerosis, periarteritis nodosa, interstitial myocarditis and hyalinization of small arteries and arterioles in various tissues and organs; the lesions are especially prominent in the heart and the cerebral cortex.[2] Similar lesions may also accompany renal hypertension in the rat,[3] thus leading to the view that desoxycorticosterone acetate may exert its action through the intermediacy of a renal pressor substance.[4]

Investigations on parabiotic rats have revealed that arterial hypertension may be produced in such a preparation by excision of three of the four kidneys, the hypertension becoming manifest in the totally nephrectomized partner.[5] Other investigators have found that even union between a normal and a bilaterally nephrectomized rat results in hypertension in the latter.[6] Again, if one of the four kidneys in a parabiotic pair is encapsulated in a gauze or a cellophane membrane, hypertension results. According to Grollman and Rule,[7] the hypertension appears in the animal with the wrapped kidney, whereas the normal partner usually maintains a normal blood pressure. Braun-Menendez and von Euler,[8] however, reported that in parabiotic union the animal with the wrapped kidney undergoes arterial hypertension only if attached to a partner having hypertension or to a nephrectomized partner. Parabiosis between a normal animal and an animal with

This study was supported by a grant from the American Heart Association.

From the Carter Physiological Laboratory, University of Texas Medical Branch.

1. (*a*) Selye, H., and Hall, C. E.: Am. Heart J. **27**:383, 1944. (*b*) Selye, H.; Hall, C. E., and Rowley, E. M.: Canad. M. A. J. **49**:88, 1943. (*c*) Selye, H., and Pentz, E. I.: Ibid. **49**:264, 1943. (*d*) Perera, G. A.; Knowlton, A. I.; Lowell, A., and Loeb, R. F.: Effect of Desoxycorticosterone Acetate on the Blood Pressure of Man, J. A. M. A. **125**:1030 (Aug. 12) 1944.

2. Selye, H.; Beland, E., and Sylvester, O.: Exper. Med. & Surg. **2**:224, 1944. Selye and Pentz.[1c]

3. Smith, C. C., and Zeek, P. M.: Am. J. Path. **23**:147, 1947.

4. Selye, H.: Stress, Montreal, Canada, Acta, Inc., 1950.

5. Jeffers, W. A.; Lindauer, M. A.; Twaddle, P. H., and Wolferth, C. C.: Am. J. M. Sc. **199**:815, 1940.

6. von Euler, U. S., and Braun-Menendez, E.: Rev. Soc. argent. de biol. **24**:362, 1948.

7. Grollman, A., and Rule, C.: Am. J. Physiol. **138**:587, 1943.

8. Braun-Menendez, E., and von Euler, U. S.: Rev. Soc. argent. de biol. **24**:355, 1948.

arterial hypertension results in the blood pressure of the latter declining to normal levels, although hypertension may be reestablished in this rat by severing the connection and permitting the animals to become separate individuals.

Inasmuch as renal hypertension has been studied by the technic of parabiosis, while desoxycorticosterone hypertension has not, and in view of the fact that it has been inferred that the latter type of hypertension is due to a renal mechanism,[4] it appeared that a study of the desoxycorticosterone induced hypertension might be profitably employed to compare the behavior of hormonal hypertension and renal hypertension under these circumstances.

MATERIALS AND METHODS

Twenty pairs of female or castrate male rats, weighing 47 to 75 Gm., were used. In each case litter mates, paired for body weight to within 5 Gm., were placed in parabiosis according to the method of Bunster and Meyer.[9] They were allowed to consume tap water and purina® laboratory chow ad libitum. One week later a 1 per cent solution of sodium chloride was substituted for the tap water. At this time two 50 mg. pellets of crystalline desoxycorticosterone acetate[10] were implanted subcutaneously in the right lateral surface of the right hand partner. In this way the desoxycorticosterone acetate was prevented from coming into direct contact with the tissues of the left hand partner. Any desoxycorticosterone, except for that which might be elaborated by its own adrenal cortex, would therefore reach the left hand partner only through the capillary anastomosis established between the pair.

Throughout the period of the experiment blood pressures were measured in the pairs once weekly, the tail-plethysmograph method of Williams, Harrison and Grollman[11] as modified by Sobin[12] being used, and the rats being unanesthetized. The animals were killed between 30 and 40 days after the desoxycorticosterone treatment was initiated, or 37 to 47 days after union was established between the pairs. At this time the kidneys, the adrenals, sections of the mesentery and the pancreas, and the ventricles of the heart were excised and placed in Bouin's fixative for weighing and subsequent histological study.

RESULTS

In 15 of the pairs studied, or 75 per cent, arterial hypertension developed only in the left hand partner, i. e., the one not treated with desoxycorticosterone. Only in five pairs, or 25 per cent, did hypertension develop in the partner which bore the implants. In no case did pairs come to autopsy in which both partners had high blood pressure or in which hypertension had failed to develop in either. In our experience hypertension produced in only the right hand partner has always occurred in pairs in which the scapular connection had separated and the attachment between the two had become reduced to a rather short pedicle of skin. The pairs then behaved essentially as independent animals. In all cases in which the connection was intimate, hypertension has developed in the left hand partner, although direct absorption of desoxycorticosterone acetate was confined to the attached parabiont. A typical record of blood pressures in such a case is given in figure 1 *A*.

9. Bunster, E., and Meyer, R. K.: Anat. Rec. **57**:339, 1933.

10. The Schering Corporation, Bloomfield, N. J., supplied the desoxycorticosterone acetate used in these studies.

11. Williams, J. R., Jr.; Harrison, T. R., and Grollman, A.: J. Clin. Investigation **18**:373, 1939.

12. Sobin, S. S.: Am. J. Physiol. **146**:179, 1946.

Curiously enough, the blood pressure was much higher in nine of the 15 cases of hypertension of the partner that did not have implants than we customarily encountered in single animals treated with this dosage of desoxycorticosterone acetate for a similar period and without unilateral nephrectomy. Blood pressure in excess of 200 mm. of mercury was attained in every instance, and in one a pressure of 275 mm. was observed. In the remaining six pairs a moderate hypertension of between 155 and 185 mm. developed. Of the five pairs in which hypertension occurred only in the right hand partner, three had blood pressures in excess of 200 mm. of mercury, whereas two showed only a moderate rise.

Rather commonly, a more or less severe hypotension has been encountered in the parabiont attached to a partner which had high blood pressure. This does not appear to have been reported hitherto in studies of hypertension in parabiotic

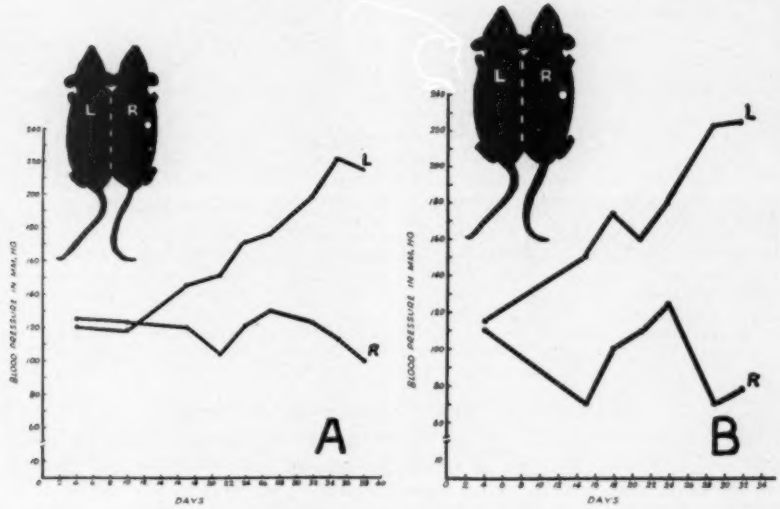

Fig. 1.—*A*, blood pressure recordings of a parabiotic pair of rats. In the right partner two 50 mg. pellets of desoxycorticosterone acetate had been implanted in the position indicated by the white dot, on the seventh day of the parabiosis. The blood pressure of this animal remained at normal levels throughout, except that it showed some decline terminally. The blood pressure of the left partner, however, quickly rose to hypertensive levels and continued to rise throughout the period of observation.

B, blood pressure recordings of a parabiotic pair of rats. The right animal received two 50 mg. pellets of desoxycorticosterone acetate on the seventh day of the parabiosis. Again the left animal became hypertensive, while the treated rat showed frank hypotension with remission and exacerbations.

rats. This manifestation has appeared in 10 of the pairs in which hypertension developed in the left hand partner, and in one of the five pairs in which the blood pressure rose only in the right. This episode of hypotension might be encountered either once or in several consecutive readings. Occasionally it persisted, usually with remissions and exacerbations, until the pair was killed. A typical case of the latter event is presented in figure 1 *B*. Inasmuch as hypotension might be

encountered in only a single reading, and the readings were spaced a week apart, it may be that hypotension occurs in parabiotic rats even more commonly than our results suggest.

At autopsy it was evident that typical nephrosclerosis, cardiac lesions and periarteritis nodosa were present in all the animals with a severe grade of hypertension, regardless of whether this was present in the partner treated with desoxycorticosterone acetate or its attached parabiont. Cardiac hypertrophy was apparent in all the hypertensive animals. Pertinent body and organ weights are given in the table, together with their standard errors calculated according to the formula

$$\text{S. E. M.} = \sqrt{\frac{d^2}{n(n-1)}}$$

Microscopic Observations.—Kidneys: Microscopic examination of the kidneys from hypertensive animals revealed a high incidence of sclerosis of the glomerular tuft capillaries and smaller arterioles. The tubules were dilated, and the lumens of many of them were filled with homogeneous hyaline casts (fig. 2 *A* to *C*).

Organ and Body Weights in 20 Pairs of Parabiotic Rats; Right Animal Treated with Desoxycorticosterone Acetate

	Left Partner Hypertensive (15 Pairs)		Right Partner Hypertensive (5 Pairs)	
Weight	Left Partner	Right Partner	Left Partner	Right Partner
Body, Gm.				
Initial	57 ± 2	57 ± 2	59 ± 4	60 ± 3
Final	133 ± 11	130 ± 11	124 ± 22	113 ± 18
Kidney, Gm.	1.006 ± 0.102	1.650 ± 0.183	1.309 ± 0.159	1.809 ± 0.253
% body weight	1.24 ± 0.07	1.28 ± 0.10	1.10 ± 0.07	1.69 ± 0.21
Heart, Gm.	0.581 ± 0.028	0.453 ± 0.044	0.463 ± 0.061	0.572 ± 0.083
% body weight	0.45 ± 0.03	0.36 ± 0.03	0.40 ± 0.03	0.50 ± 0.02
Adrenals, mg.	37.8 ± 3.1	29.5 ± 2.1	34.8 ± 1.7	42.4 ± 8.2

Occasionally perivascular or peritubular collections of small round cells of the small lymphocyte type were observed. Vascular lesions were graded arbitrarily on a scale of 1 + to 3 +. In those animals in which an extreme degree of hypertension had developed, the severity of the lesions was 3 +, whereas it was only 1 + in those with mild hypertension. Some of the latter, however, were without visible vascular lesions.

In addition to the vascular lesions, changes strikingly similar to those reported by Selye and Stone [13] in the "endocrine kidney" were observed. In five of the 15 pairs in which hypertension developed in the left animal, the kidneys of the right partner were found to contain small, shrunken glomeruli, fewer than in a corresponding section from a normal kidney. In addition, the visceral and parietal layers of Bowman's capsule had fused, thus causing the glomerulus to merge with the contiguous parenchyma. In three of these cases cross sections taken through the center of the kidney and examined under low magnification revealed only two or three poorly defined glomeruli in the entire section. The cortex showed marked thinning, and there were evidences of atrophic changes in the proximal convoluted tubules (fig. 2 *D*). In two cases these changes were observed in

13. Selye, H., and Stone, H.: J. Urol. **56**:399, 1946.

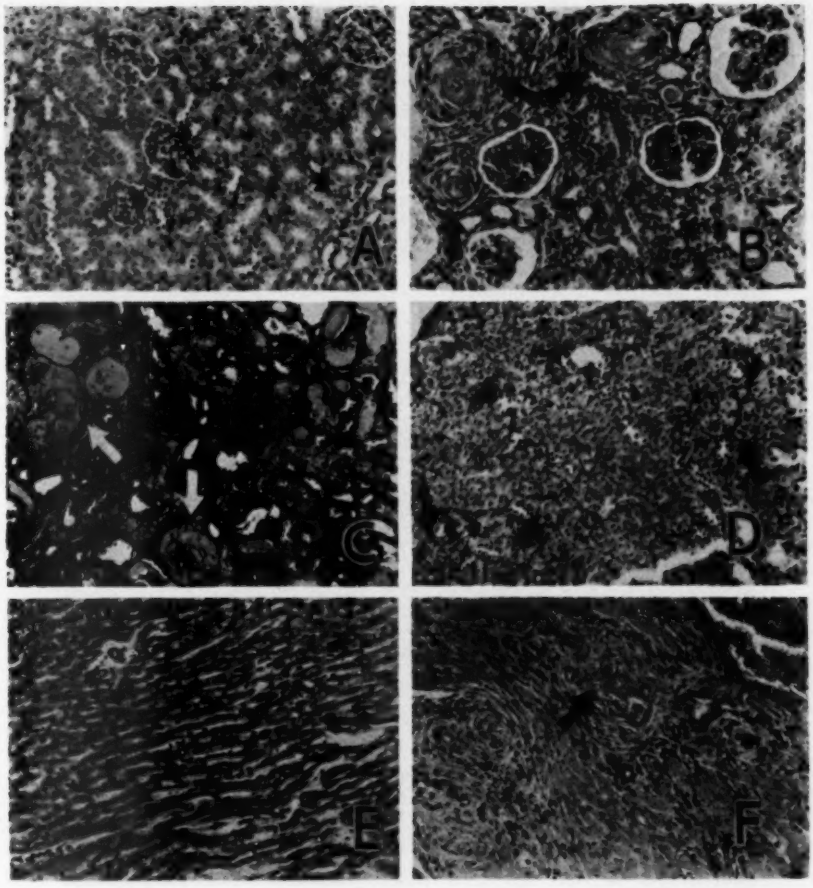

Fig. 2.—*A*, section of a kidney taken from the desoxycorticosterone-treated rat of a parabiotic pair which had been in parabiosis for 36 days. This rat had been treated for 29 days. The tissue appears to be entirely normal. × 115.

B, section of kidney from the partner of the above rat. The arrows indicate arterioles in which the walls have undergone hyaline transformation and in which swelling has led to almost complete obliteration of the lumens. The subcapsular space of the glomerulus in the upper right hand corner contains an amorphous transudate. × 115.

C, section of kidney from a rat in parabiosis with a desoxycorticosterone-treated partner for 34 days. The latter had received implants of the compound 27 days previously. Glomeruli that have undergone almost complete hyaline necrosis are indicated by arrows. The tubules contain hyaline casts. × 115.

D, changes suggestive of "endocrine" transformation in the kidney of a rat in parabiosis for 39 days and treated with desoxycorticosterone for 32 days. The glomeruli, indicated by arrows, have fused with the surrounding parenchyma because of merging of the visceral and parietal layers of Bowman's capsule with obliteration of the subcapsular space. Note disappearance of tubular lumens and atrophy of tubules. × 115.

E, section of the heart from the animal described in *A*. × 115.

F, section of the heart from the animal attached to the above (same rat as in *B*). Note the hyalinized and necrotic wall of the arteriole indicated by the arrow. Others may be seen in the section. Surrounding these there is a granulomatous proliferative reaction containing many inflammatory and giant cells. × 115.

The photomicrographs in figures 2, 3, 4 and 5 were made by the department of medical illustration of the University of Texas. The sections were stained with hematoxylin and eosin.

the left animal, the one showing hypertension. In such kidneys the renal pyramid consisted of almost solid tissue, the lumens of the collecting ducts being obliterated (figs. 3 and 4). In 10 of the 15 rats bearing pellets of desoxycorticosterone acetate, in which hypertension developed in the attached partner, the kidneys were essentially normal histologically in the former despite the presence of desoxycorticosterone (fig. 2 *A*). Some enlargement of tubules, resembling a renotrophic action, was observed; and in a few, individual glomeruli were observed which seemed to fuse with the adjacent parenchyma, producing the appearance of an "endocrine nephron."

Heart: Left ventricular hypertrophy was encountered in all the hearts from hypertensive animals, the degree being dependent on the severity and the duration of the hypertension. In addition to this, marked degeneration of myocardial fibers with round cell infiltration of the stroma and proliferation of connective tissue

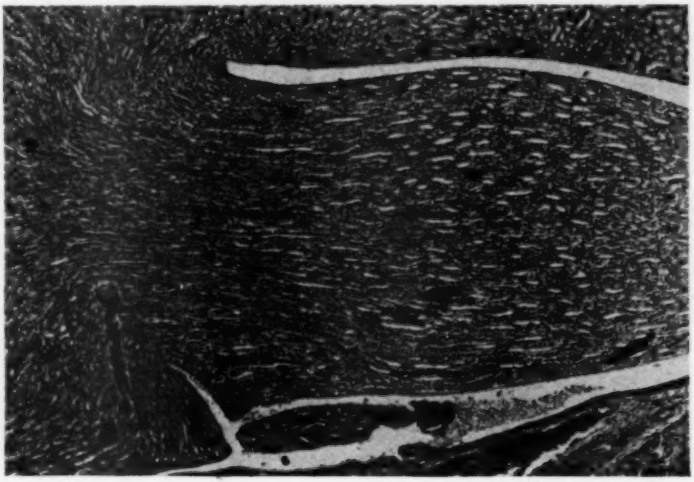

Fig. 3.—Section through a renal pyramid of a rat in parabiosis for 38 days and treated with desoxycorticosterone acetate for 31 days. Normal histological structure is maintained. × 37.

elements were observed to occur in those cases in which the hypertension had been severe. In such hearts extensive hyalinization of the walls of the small arterioles was frequently observed, and about such vessels a proliferative reaction, consisting of large polymorphonuclear giant cells and histiocytes, forming a structure suggestive of the "Aschoff" nodule was commonly observed (fig. 2 *E* and *F*).

Pancreas and Intestine: These organs will be considered together, since the vascular lesions in each were of essentially the same type. Periarteritis nodosa of the mesenteric and pancreatic arteries was the most prominent feature in all the rats in which severe hypertension occurred. In both areas, arterial vessels with hyalinized or necrotic walls were frequently encountered in sections. A marked periarterial inflammatory reaction was commonly observed, characterized by an invasion of the arterial walls in which leukocytes, both polymorphonuclear and mononuclear, and giant cells participated (fig. 5).

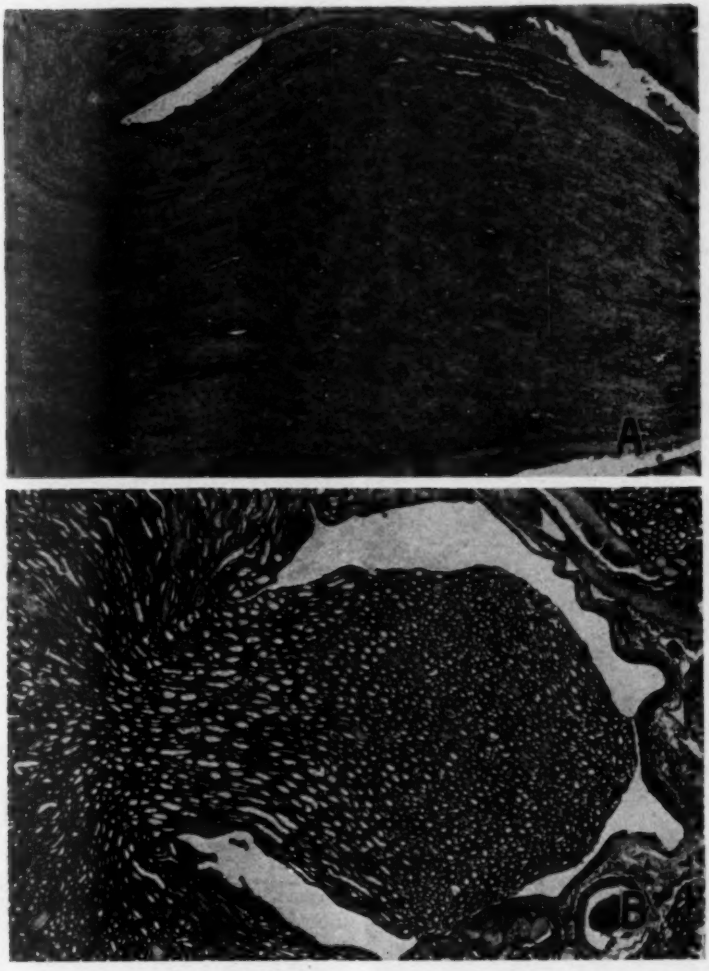

Fig. 4.—*A*, section through a renal pyramid of a rat in parabiosis for 37 days and treated with desoxycorticosterone acetate for 30 days. The collecting tubules are greatly reduced in number, the section giving the appearance of almost solid tissue. This kidney had seemingly undergone almost complete "endocrine" transformation. Normal glomeruli were entirely absent, and structures even remotely resembling glomeruli were identified only with difficulty. × 37.

B, section through a renal pyramid of a kidney of the partner of the rat described above. Intense nephrosclerosis had developed. Note the distortion of shape, due to distention of the tubules with casts and fluid and also to contracture of the kidney as a result of cortical scarring. × 37.

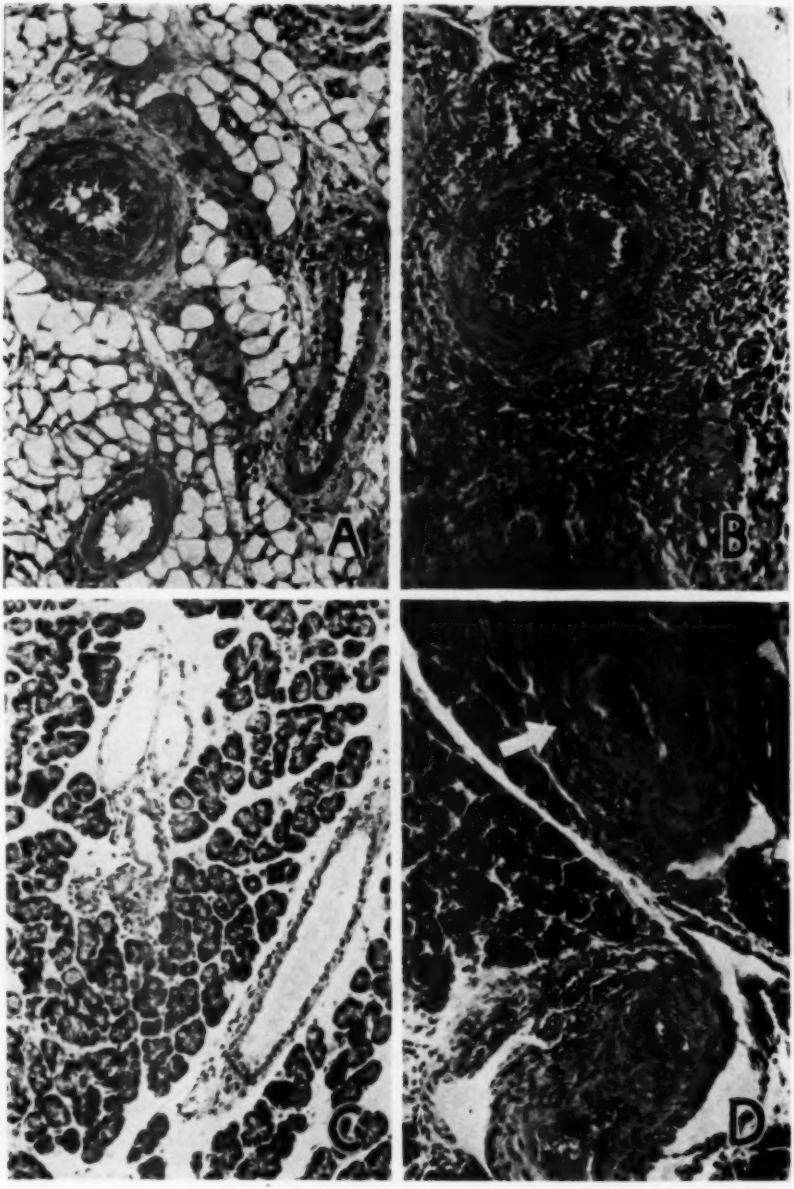

Fig. 5.—*A*, transverse section through the mesenteric artery of a rat in parabiosis for 38 days and treated with desoxycorticosterone acetate for 31 days. The structure is normal. × 144.

B, transverse section through the mesenteric artery of the untreated partner of the above rat. The upper segment of the wall has undergone hyaline necrosis. The wall of the vessel and the surrounding tissue are heavily invaded by inflammatory cells, the typical lesion of periarteritis nodosa. × 144.

C, section of pancreas from the rat described in *B*, showing normal histologic structure. × 144.

D, periarteritis nodosa in the pancreas of the partner of the rat described in *B*. Note that the arterial wall, indicated by the arrow, had undergone hyaline necrosis and been invaded and surrounded by inflammatory cells. × 144.

Such lesions were observed to occur in all animals in which hypertension was severe, and thus they appeared in the untreated litter mates three times as commonly as they appeared in the treated ones, and were absent from the latter in all instances in which satisfactory union between the paired animals was maintained.

Adrenals: The adrenals of the rat in which pellets of desoxycorticosterone acetate had been implanted were smaller than those of the partner only when the treated animal had normal or low blood pressure. When, however, hypertension developed in an animal treated with desoxycorticosterone acetate, the adrenals in this animal were, on the average, larger than those of the partner. Although this was not an invariable rule, it appeared that in general the adrenals were largest in whichever of the animals had high blood pressure.

COMMENT

In planning the experiment we assumed a priori that, disregarding hypotension, which was an unexpected finding and probably is a manifestation of the parabiotic state,[14] four possibilities existed: (1) Neither of the parabiotic litter mates would become hypertensive, (2) both would become hypertensive, (3) the treated partner but not the other would become hypertensive, (4) hypertension would develop not in the treated rat but in its partner. Any of these possibilities except the last, which turned out to be the typical response, could easily be explained.

The first possibility would agree with the findings of Braun-Menendez on parabionts with renal hypertension, and the conclusion would be that the partner not receiving desoxycorticosterone acetate, being normal, was able to detoxify the pressor substance reaching it from the treated animal, thus reducing the amount circulating and maintaining the normal blood pressure. The second contingency could be interpreted as indicating that desoxycorticosterone, or a pressor substance produced as a result of desoxycorticosterone treatment, was present in sufficient amounts in both rats to cause hypertension. The third eventuality would agree with the results observed by Grollman and Rule on parabiotic rats with renal hypertension and would suggest that desoxycorticosterone or a renal pressor substance elicited by it, while reaching effective concentrations in the treated animal, did not do so in the other of the pair. The fourth and actual result does not seem to be susceptible of any simple explanation.

It would seem that exogenous desoxycorticosterone cannot be held solely accountable for the hypertension and accompanying lesions observed in these experimental animals, since both were most commonly encountered in the partner not directly treated with desoxycorticosterone acetate. Indeed, in 75 per cent of the rats treated with this compound neither hypertension nor vascular lesions were observed, whereas in the same pairs they were present in the rats which did not have implanted pellets of desoxycorticosterone acetate.

Experiments have shown that approximately 50 cc. of blood per day crosses the capillary inosculation established between the animals of a parabiotic pair.[15] Data pertaining to the behavior of adrenal steroids under these circumstances are not available, but it is known that androgens and estrogens do not effectively cross

14. Hall, C. E.: Unpublished data.

15. Van Dyke, D. C.; Huff, R. L., and Evans, H. M.: Stanford M. Bull. **6**:271, 1948.

from the treated partner to its twin. Approximately 50 units of androgenic or 80 units of estrogenic steroid must be injected into one partner in order to produce 1 unit of effect in the other.[16] Bearing these limitations in mind, we find no conceivable way in which the hypertensive left partner might be subjected to a greater amount of circulating desoxycorticosterone than the right partner which receives the implants, unless its supply be of endogenous origin.

Nor does it seem reasonable to suppose that the smaller amount of desoxycorticosterone reaching the left animal more effectively induces hypertension than the larger quantities to which the right hand animal is subjected, since clinical and experimental evidence indicates that larger doses of desoxycorticosterone acetate produce hypertension more readily and more severely than smaller doses. The severe hypertension and vascular lesions which occur in the left hand partners in such a short period of time cannot be accounted for solely by the minute quantities of desoxycorticosterone which they must receive through the vascular inosculation.

Similar objections pertain to the theory of a renal pressor substance passing from the desoxycorticosterone-treated rat to its parabiont. If it is supposed that desoxycorticosterone causes the kidney to release a pressor material, then it is difficult to explain why the right hand animal remains normotensive or hypotensive in the majority of cases. Assume that a pressor substance is produced in the treated animal; then, if the mere 2 cc. of blood which is hourly exchanged between the two animals carries enough pressor substance to make the partner hypertensive, why does not the donor animal with presumably a much greater quantity of pressor substance become hypertensive? More commonly the rat in which the pellets have been implanted is hypotensive. Then, too, these results are at variance with those reported for parabiotic animals in which hypertension of known renal origin had been produced.

We have no explanation for the fact that hypertension occurred more frequently in the left hand partner than in the desoxycorticosterone-treated right parabiont. An increased blood volume in the left hand animal at the expense of the right would cause hypertension in the former and hypotension in the latter, but we have no proof of such an occurrence as yet. The parabiotic pairs reported have been healthy animals, growing well throughout the experimental period.

In the five cases in which the desoxycorticosterone-treated parabiont became hypertensive, the kidneys of that animal were always larger than those of the partner. In the remaining 15 cases, in which the nontreated parabiont became hypertensive, this uniformity was not observed. Both administration of desoxycorticosterone acetate and hypertension tend to increase the renal mass, and since each exerts its influence in one of the pair in 75 per cent of the cases, the two opposing factors tend to equalize the renal mass of the two partners. The kidneys of the former seem to reflect a renotrophic action; those of the latter, a nephrosclerotic one.

The renal changes similar to those occurring in the "endocrine kidney" which were observed may result from the episodes of hypotension occurring in animals bearing such kidneys. This probably reduces the hydrostatic pressure within the afferent arterioles of the glomeruli below that necessary for maintaining their integrity.

16. Shipley, E. G.; Meyer, R. K., and Biddulph, C.: Am. J. Physiol. **140**:230, 1943.

Cardiac hypertrophy, as would be expected, is always present in the hypertensive rat regardless of which one received the desoxycorticosterone acetate.

Adrenal size was reduced by desoxycorticosterone only when the treated partner did not become hypertensive. In hypertensive animals in which desoxycorticosterone acetate had been implanted, the cortex was usually enlarged above that of the twin.

SUMMARY

Of 20 pairs of parabiotic rats in which pellets of desoxycorticosterone acetate had been implanted in the right hand partner, hypertension developed in the left hand animal in 15, or 75 per cent, and in the remaining five, or 25 per cent, the blood pressure rose in the animal receiving the implants. The latter five pairs did not maintain a good vascular union.

Hypotension was frequently observed in animals attached to hypertensive partners, regardless of whether or not they had been treated with desoxycorticosterone acetate.

Nephrosclerosis, periarteritis nodosa and cardiac lesions were encountered only in severely hypertensive animals, regardless of which of the parabionts bore the pellet of desoxycorticosterone acetate.

Changes reminiscent of those occurring in the "endocrine kidney" were observed in five rats treated with desoxycorticosterone acetate and in two attached to such rats.

The significance of these observations is discussed.

EXPERIMENTAL HYPERSENSITIVITY IN THE RABBIT

The Cellular Localization of Soluble Azoproteins (Dye-Azo-Human Serum Albumins) Injected Intravenously

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THE PRESENT study was made to determine whether there might be any correlation between the tissue and cellular localization of a protein antigen, the cells suspected of producing antibodies, and the sites of hypertensive lesions. For this, labeled proteins are convenient.

In working out the details of the antigen-antibody reaction in vitro, proteins have been labeled variously with chemicals, including iodine [1] and arsenic,[2] azodyes [3] and radioactive isotopes.[4] A number of immunologic studies employing labeled proteins have been made in vivo.[5]

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This work was supported by grants recommended by the Panel on Hematology and by the National Cancer Institute, from the National Institutes of Health, United States Public Health Service.

This is one of a series of clinical, pathological and immunologic studies on the proteins of blood and tissues, in which proteins prepared by methods developed in the department of physical chemistry of Harvard Medical School were used.

1. Wormall, A.: J. Exper. Med. **51**:295, 1930.

2. Haurowitz, F., and Breinl, F.: Ztschr. f. physiol. Chem. **205**:259, 1932. Boyd, W. C.: Fundamentals of Immunology, New York, Interscience Publishers, Inc., 1947, p. 58.

3. (*a*) Heidelberger, M., and Kendall, F. E.: J. Exper. Med. **59**:519, 1934; (*b*) **62**:467, 1935. (*c*) Landsteiner, K.: The Specificity of Serological Reactions, Cambridge, Harvard University Press, 1945.

4. Boursnell, J. C.; Dewey, H. M.; Francis, G. E., and Wormall, A.: Nature, London **160**:339, 1947. Francis, C. E., and Wormall, A.: Biochem. J. **42**:469, 1948.

5. (*a*) Coons, A. H.; Creech, H. J.; Jones, R. N., and Berliner, E.: J. Immunol. **45**:159, 1942. (*b*) Coons, A. H., and Kaplan, M. H.: J. Exper. Med. **91**:1, 1950. (*c*) Fine, J., and Seligman, A. M.: J. Clin. Investigation **22**:285, 1943; (*d*) **23**:720, 1944. (*e*) Gitlin, D.: Proc. Soc. Exper. Biol. & Med. **74**:138, 1950. (*f*) Heidelberger, M.; Kendall, F. E., and Soo Hoo, C. M.: J. Exper. Med. **58**:137, 1933. (*g*) Heidelberger, M.; Treffers, H. P.; Schoenheimer, R.; Ratner, S., and Rittenberg, D.: J. Biol. Chem. **144**:555, 1942. (*h*) Landsteiner.[3c] (*i*) Libby, R. I., and Madison, C. R.: J. Immunol. **55**:15, 1947. (*j*) Pratt, H. N., and Gregersen, M. I.: Ibid. **40**:163, 1941. (*k*) Pressman, D.; Hill, R. F., and Foote, F. W.: Science **109**:65, 1949. (*l*) Sabin, F. R.: J. Exper. Med. **70**:67, 1939. (*m*) Seligman, A. M., and Fine, J.: J. Clin. Investigation **22**:265, 1943. (*n*) Smadel, J. E., and Swift, H. F.: J. Exper. Med. **74**:345, 1941. (*o*) Smetana, H.: Ibid. **45**:619, 1927. (*p*) Warren, S., and Dixon, F. J.: Am. J. M. Sc. **216**:136, 1948; (*q*) Am. J. Path. **25**:812, 1949.

In order to obtain more accurate quantitative and qualitative data on the fate of intravenously injected soluble protein antigens, a series of experiments was planned using different purified proteins labeled variously with several azodyes and with radioactive iodine. Studies with human serum albumin and bovine serum gamma globulin labeled with radioiodine are being reported elsewhere.[6]

The present paper reports the visible tissue and cellular localization of Evans blue-azo-human serum albumin (EB-HA), orange G-azo-benzidine-azo-human serum albumin (OG-B-HA) and acriflavine-azo-human serum albumin (A-HA) injected intravenously into rabbits. For control purposes the dyes alone, as well as india ink, were also injected into rabbits. The immunologic behavior of the protein antigens used in these experiments, as studied by serial measurements of their blood concentrations following injection,[7] is reported separately.[8]

MATERIALS AND METHODS

Male albino rabbits averaging about 1,800 Gm. in weight were given a single injection of the dye-protein in isotonic solution of sodium chloride (10 cc. per kilogram) in the right ear vein and were bled at intervals from the left ear vein. The azoproteins were prepared according to the method described by Heidelberger, Kendall and Soo Hoo [8f] producing a diazo linkage. As the proteins contained varying amounts of water and some protein was lost in the synthesis, the final solutions were less than the intended 10 per cent by weight. Nitrogen determinations by the micro-Kjeldahl method gave the following concentrations of protein in the solutions injected: EB-HA, 7.78 per cent; OG-B-HA, 8.69 per cent; A-HA, 4.58 per cent.

Two animals in each series were killed one day after receiving the injection in order to determine the early localization of antigen before antibody formation. Originally it was planned to kill half of the remaining animals just before the proteins disappeared from the circulation and the other half about five days later. However, since intercurrent diseases developed in several of the rabbits, the animals were killed somewhat earlier than was planned. Consequently, some protein was found at death in the blood of all but one of the animals. (In that one no specific antibodies could be detected.)

An amount of each dye equivalent to the amount attached to the protein was dissolved in isotonic sodium chloride solution, and 10 cc. per kilogram was injected intravenously in the control animals. Two rabbits received orange G, two acriflavine and two Evans blue. All were killed 24 hours later. In addition, two rabbits received 6 cc. of Higgins waterproof india ink, previously centrifuged to remove the larger particles.

All animals were killed with a blow on the head and were bled immediately from the carotid arteries. The tissues were fixed in Zenker-formaldehyde solution and in solution of formaldehyde U. S. P. diluted 1:10. After washing, the tissues fixed in Zenker-formaldehyde solution were stored in 80 per cent alcohol. The interval until the preparation of slides varied from a few days to six months, but no differences were noted in dye content of the tissues from similarly treated and killed animals. Hematoxylin-eosin sections were prepared in the usual manner. Both Evans blue and acriflavin could be found in these sections, but all the dyes were much more easily found in sections counterstained lightly with a contrasting color. For this the tissues from the EB and EB-HA animals were washed in 95 per cent alcohol to remove the iodine and were stained lightly for 10 minutes in alcoholic carmine. The tissues from the OG-B-HA and OG treated animals were counterstained lightly with Harris' hematoxylin diluted. The tissues from animals given india ink injections were stained with alcoholic carmine.

6. Latta, H.: To be published.

7. Gitlin, D.: J. Immunol. **62**:437, 1949. Gitlin, D.; Davidson, C. S., and Wetterlow, L. H.: Ibid. **63**:415, 1949.

8. Gitlin, D.; Latta, H., and Janeway, C. A.: To be published.

OBSERVATIONS

In all sections from the rabbits into which azoproteins had been injected, the dye was seen only as small, round granules in the cytoplasm of various cells. A somewhat different appearance was noted in the tissues of the control animals receiving Evans blue, described below. As there were only slight differences between the tissues of rabbits killed one day after the injection of azoprotein and those killed or dying later, the findings in each series will be described together. There was a fairly regular, though not invariable, tendency for the number of granules seen in the cells of a given organ to decrease with time after the first day, while the individual granules seemed to become coarser and showed a deeper specific color corresponding to the injected azoprotein. The observations on all series are summarized in the table.

Tissue Localization of Intravenously Injected Substances

	Experimental Rabbits			Controls			
	EB-HA	OG-B-HA	A-HA	EB	OG	A	India Ink
Circulatory macrophages	0	0	+	±	0	0	++
Spleen	+	±	+++	T	0	0	++++
Marrow	+	+	+++	T	0	0	++++
Liver:							
(a) Kupffer cells	+	±	+++	T	0	0	++++
(b) Liver cells	+	±	+	0	0	0	+
Adrenal:							
(a) Endothelium in reticular zone	..	..	+	0	0	0	+
(b) Endothelium in medulla	+	T	..	T	0	0	..
(c) Cortical cells	0	0	+	0	0	0	±
Lymph node	+++	++	+	T	0	0	+
Intestines	+	T	T	0	0	0	T
Muscle	T	T	0	0	0	0	T
Esophagus	+	±	0	0	0	0	T
Heart (valves)	++	++	++	0	0	0	+
Lungs (alveoli)	±	0	+	0	0	0	++
Kidney:							
(a) Glomeruli	0	0	+	0	0	0	+
(b) Proximal tubules	++	±	0	+	0	0	0
Synovial membrane	+	+	T	T	0	0	±

T = a trace, found after careful searching through many microscopic fields, in one or several rabbits.
± = found after searching through a few high power fields.
+ = found in almost every high power field.
++ to ++++ = found in increased amount.

The need for caution in identifying hypersensitivity lesions in the presence of naturally occurring diseases has been emphasized.[9] No lesions were found that could be attributed exclusively to hypersensitivity resulting from the experimental injection of foreign protein. Except for the active myocarditis and epicarditis in many of the animals, there was no definite or constant relationship between the observed lesions and the visualized site of azoprotein localization in these experiments.

EXPERIMENT 1.—*Evans Blue-Azo-Human Serum Albumin.*—Ten animals were used. Two were killed after one day. One died on the second day and one on the third day, and a third animal was killed on the seventh day. One died with colitis on the eighth day; the four remaining animals were killed on the eighth day. All tissues were grossly blue. In all cases in which urine was observed, it had the usual yellow color.

9. (*a*) Hawn, C. V. Z., and Janeway, C. A.: J. Exper. Med., **85**:571, 1947. (*b*) More, R. H., and McLean, C. R.: Am. J. Path. **25**:413, 1949. (*c*) Rich, A. R., and Gregory, J. E.: Bull. Johns Hopkins Hosp. **72**:65, 1943.

Microscopically, the blue granules were found predominantly in cells of the macrophage system in all tissues throughout the body: in connective tissue of muscle, neck, intestines, thymus, pancreas, testis; in adventitia of blood vessels; in the splenic pulp (but not in the malpighian bodies); in macrophages and endothelial cells of the bone marrow, and in endothelial cells of the adrenal medulla. Since in each of the following organs the localization is of special interest, it will be described in more detail.

Lymph Nodes: There was a variation in the amount of dye contained in nodes from different areas, in nodes of the same area, and even in different portions of the same node. The more involved nodes were considered for comparison. The lymph nodes showed a greater concentration of the dye protein than any other tissue.

Within the lymph nodes the distribution of the blue granules changed with time. In rabbits killed one day after the injection of the dye fine blue granules appeared predominantly in the flattened littoral cells lining the lymph sinuses, especially in the medullary portion of the node (fig. 1*A*). Some stellate reticular cells of the sinusoids also contained granules. The lymphoid tissue and the macrophages contained in it were free of the blue granules. In the rabbits killed after eight days, on the other hand, the littoral cells were practically free of blue granules, while the adjacent rounded macrophages in the lymphoid tissue were filled with large, coarse granules (fig. 1*B*). The cortex was relatively free of the dye, and none was found in the germinal or reaction centers. Such a change was found only in the lymph nodes of the rabbits that received EB-HA and was not observed in the animals given other azoproteins.

Heart: Here the commonest site of concentration of the dye granules was in the valves. Cells containing blue granules were found in both the anterior and the posterior leaflet of the mitral valve from the tip to the valve ring (fig. 1*D*). In some valves the distribution was focal, while in others it was diffuse. The cells containing dye were invariably present in the atrial layer of the valve, usually near the surface. The central connective tissue plate and the thin endocardial layer on the ventricular surface were invariably free of the granules. In the tricuspid valve, the three layers were less distinct, but the dye protein was similarly found in the atrial layer of endocardium. Conversely, in the pulmonic and aortic valves the sinus endocardium is thin and closely applied to the central connective tissue plate, with the ventricular layer being thicker and having looser connective tissue fibers. It was in this ventricular endocardial layer that the blue granules were found.

Large amounts of dye were also found in macrophages in the endocardium around the chordae tendineae (fig. 2*A*) or in the endocardium of the ventricles (fig. 2*B*). In the myocardium the dye was much less concentrated, occurring mainly in macrophages of the connective tissue septums adjacent to blood vessels (fig. 2*D*). It was frequently concentrated in the epicardium (fig. 2*C*).

Although all gradations of myocarditis and pericarditis were observed in the control and experimental rabbits of all series, most of the animals had only a rare focal accumulation of mononuclear cells. Extensive active inflammatory lesions with polymorphonuclear granulocytes and macrophages filled with the specific blue granules were also found in a few hearts. Scarring had taken place in some. More macrophages containing colored granules were associated with acute lesions of the heart than with chronic ones.

Kidney: The proximal convoluted tubules regularly contained blue granules in the central and basilar portions of the cytoplasm. The glomeruli, the loops of Henle, the distal convoluted tubules and the collecting tubules were free of dye (fig. 3*A*). Grossly and under low power magnification the cortex had a radially streaked appearance due to the absence of dye from the medullary rays. Macrophages throughout the kidney contained the dye and were especially prominent in tissue about the pelvis. Few dye-containing macrophages were found about the ureters.

Synovial Membrane: Oval and spindle-shaped cells of the subsynovial tissues of the knee regularly contained blue granules in considerable concentration (fig. 3*B*). In addition, many of the synovial lining cells themselves appeared to contain blue granules.

Lungs: In all lungs, dye was found in the macrophages in the adventitia of the pulmonary arteries and around the bronchi outside the smooth muscle (fig. 3*C*). Its presence in the

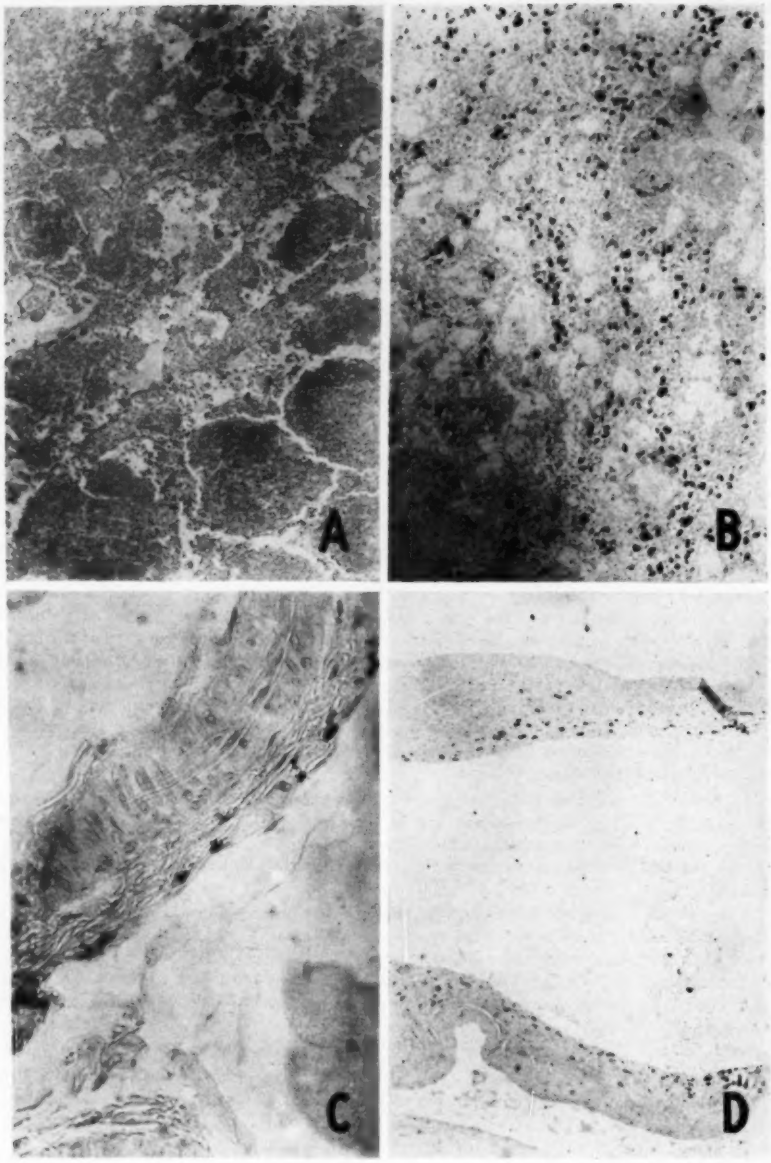

Fig. 1.—*A*, lymph node, Evans blue-azo-human serum albumin (EB-HA), rabbit 37, after one day; × 90. The dark (blue) granules are largely in the cells lining the sinusoids of the medulla.

B, lymph node, EB-HA, rabbit 45, after eight days; × 90. A few littoral cells contain (blue) granules, but the adjacent macrophages are loaded with them.

C, gastrocnemius, EB-HA, rabbit 45, after eight days; × 450. Cells containing the colored granules lie in the adventitia of a small artery in the muscle.

D, mitral valve, EB-HA, rabbit 44, after eight days; × 90. Macrophages and fibroblasts containing colored granules are found in both leaflets, entirely in the atrial layers.

Since the tissues were purposely counterstained very lightly to increase the contrast of the azo dyes used as labels, the photomicrographs in figures 1 to 6 necessarily do not reveal cellular detail with the usual clarity. In all of them the injected azoprotein (or azo dye component) appears as dark granules against the light background of the tissues.

All the photomicrographs used in this article were made by Mr. John Carabitses.

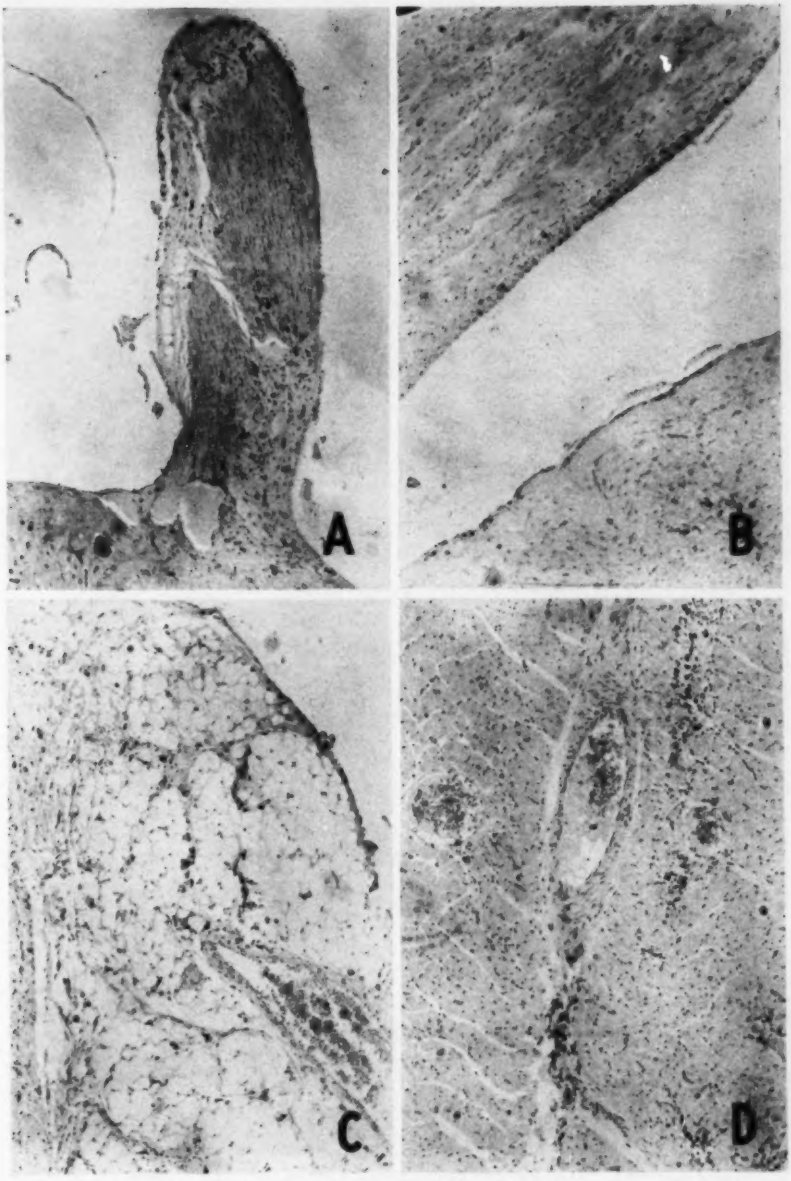

Fig. 2.—*A*, chorda tendinea of mitral valve, EB-HA, rabbit 36, after one day; × 130. Granule-filled cells are found especially in the covering endocardium.

B, endocardium, EB-HA, rabbit 42, after eight days; × 400. The granules are in cells just beneath the endothelium.

C, epicardium, EB-HA, rabbit 43, after eight days; × 72. Foci of macrophages with blue granules may be found in the epicardium. In this animal the number of macrophages is increased by an epicarditis.

D, myocardium, EB-HA, rabbit 43, after eight days; × 78. Macrophages containing the blue granules are found especially in the perivascular connective tissue and in foci of myocarditis.

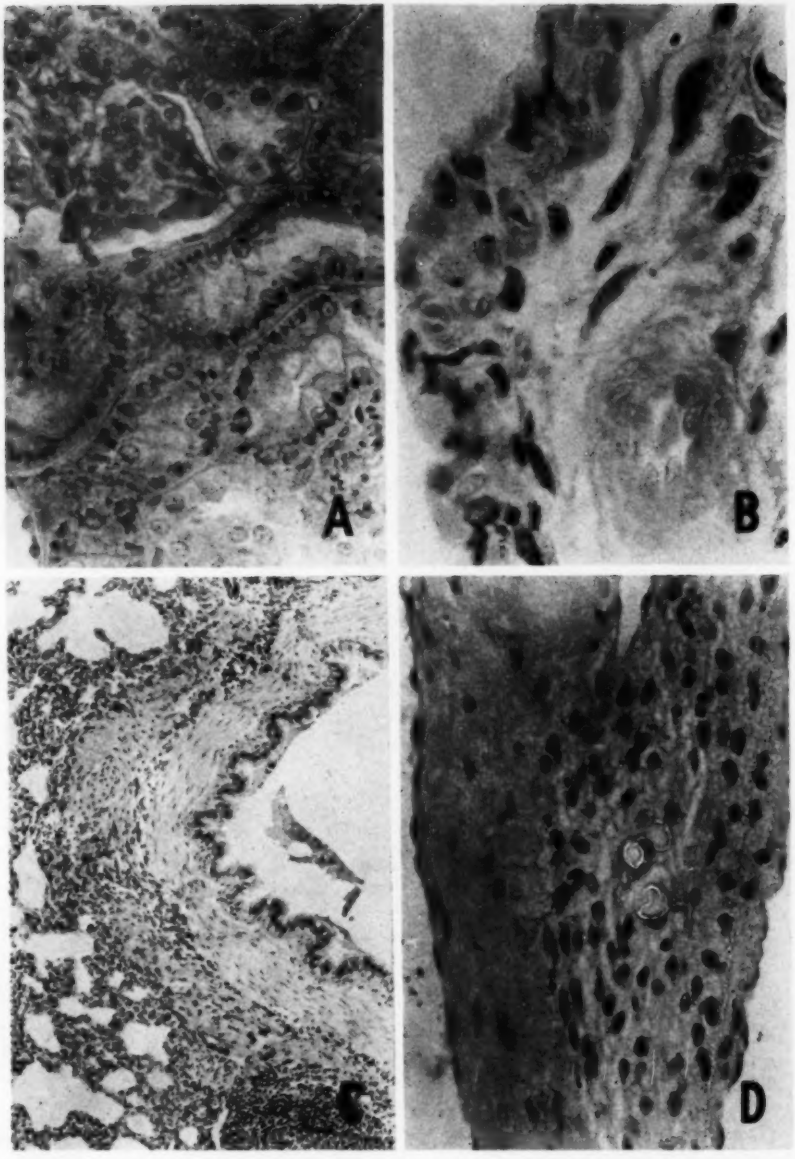

Fig. 3.—*A*, kidney, Evans blue-azo-human serum albumin (EB-HA), rabbit 36, after one day; × 520. The blue granules appear in the central portion of the epithelial cells of the proximal convoluted tubules. The remainder of the tubular system and the glomeruli are free of the blue granules.

B, synovial membrane, EB-HA, rabbit 37, after one day; × 1080. Macrophages and possibly fibroblasts and synovial lining cells contain the granules.

C, lung, EB-HA, rabbit 42, after eight days; × 96. In the lung, macrophages containing the granules are found most frequently just outside the smooth muscle of the bronchial wall (shown here) and in the adventitia of the large vessels.

D, mitral valve, orange G-azo-benzidine-azo-human serum albumin (OG-B-HA), rabbit 29, after seven days; × 320. Many cells with the dark (red) granules are in the atrial layer of the valve.

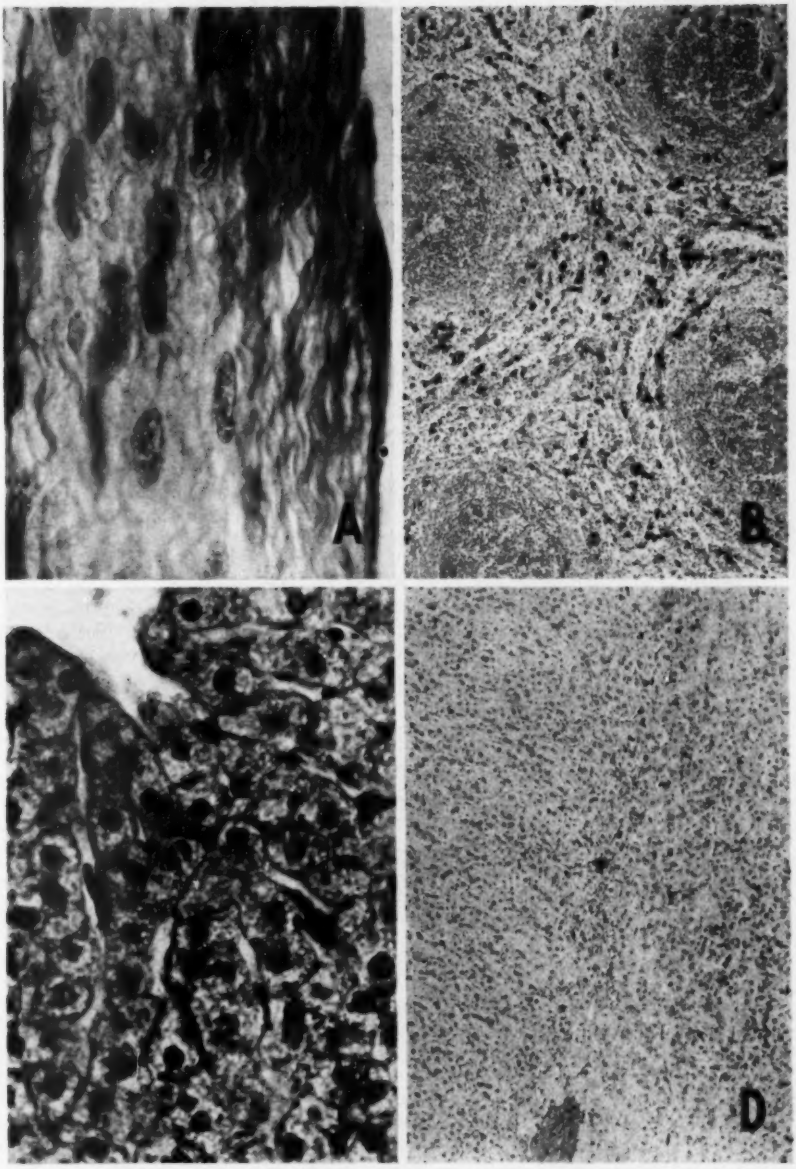

Fig. 4.—*A*, mitral valve, OG-B-HA, rabbit 29, after seven days; × 1,080. An Anitschkow cell or "myocyte" (to the left of the center of the field) has its cytoplasm filled with the dark (red) granules.

B, spleen, acriflavine-azo-human serum albumin (A-HA), rabbit 5, after eight days; × 87. The macrophages in the cords of the splenic pulp contain the major portion of the dark (amber) granules. No colored granules are seen in the germinal centers or in the lymphocytes, and relatively few are found in macrophages in the surrounding zone of lymphocytes.

C, liver, A-HA, rabbit 7, after eight days; × 550. Kupffer cells are loaded with the dark granules. Amber granules are also scattered through the liver cells themselves and appear here as single black dots.

D, adrenal, A-HA, rabbit 4, after eight days; × 120. The endothelial cells of the reticular zone show a specific accumulation of the granules. The left half of the picture shows cortex and the right half medulla.

parenchyma within macrophages of the alveolar wall was much less regular, frequently being quite patchy. In some lungs, only a few scattered parenchymal macrophages contained the granules.

Liver: Moderate amounts of dye were present in the Kupffer cells. Some granules were also found scattered through the epithelial liver cells themselves, several to a cell.

Evans Blue Alone.—In the two rabbits killed one day after injection of the dye, the kidneys were the only organs to show an appreciable amount of dye. They were practically identical with the kidneys of the EB-HA-treated animals. The only other sites in which the blue granules could be found were in rare endothelial cells of the liver and the adrenal and in one or two macrophages in the synovial membrane, the adventitia of the aorta, the spleen and lymph nodes.

EXPERIMENT 2.—*Orange G-Azo-Benzidine-Azo-Human Serum Albumin.*—Ten rabbits were used. Two were killed after 1 day, four after 7 days, and two after 10 days. One died on the fourth day and one on the ninth day. The OG-B-HA was found in the same sites as EB-HA, but the number of cells containing the dye was usually much less (table). In many of the tissues no dye was found at all.

Some dye-containing macrophages were present around the major bronchi and vessels, while none were found in the alveoli of the lungs. Similarly, dye was regularly present in the macrophages of the renal pelves, although dye was found only in portions of some of the proximal convoluted tubules in both of the rabbits killed after one day and in the rabbit dying after nine days. In the heart, especially in the valves, there was only slightly less involvement than with EB-HA (fig. 3 *D*). In these sections the cell types containing dye could be more readily distinguished. While many of the cells with dye had all the morphological characteristics of macrophages, including the darker nuclei with a heavy membrane and coarser chromatin, many cells were seen which lacked one or more of the features which distinguish fibroblasts from macrophages.[10] In fact, some of the cells were morphologically indistinguishable from fibroblasts. The Anitschkow cells or "myocytes" were found to concentrate the dye in granular form in their cytoplasm (fig. 4 *A*).

Orange G Alone.—No dye was found microscopically in any of the tissues one day after its intravenous injection in the two animals used.

EXPERIMENT 3.—*Acriflavine-Azo-Human Serum Albumin.*—Seven rabbits received injections. Two were killed after one day and five after eight days. The behavior of A-HA was in pronounced contrast to that of EB-HA and OG-B-HA, both quantitatively and qualitatively. The amber granules of the dye were quite prominent in the circulating macrophages of the two rabbits killed after one day, but in eight days they had disappeared from the circulation of all but one animal. In addition, a few circulating polymorphonuclear granulocytes contained some granules, but in smaller quantity. The macrophages of the spleen (fig. 4 *B*) and marrow and the Kupffer cells of the liver (fig. 4 *C*) were engorged with large amounts of the dye. The amber color was visible even in the finest granules and could easily be distinguished from the lighter yellow of hemosiderin, which was frequently present in the sections. Iron stains confirmed the distinction. To demonstrate the endothelium lining the sinusoids of the splenic pulp, modified Lee-Brown stains were made. These showed that by far the largest portion of the amber granules was in the macrophages of the pulp, and that relatively little was in the cytoplasm of the endothelial cells. In the adrenal the A-HA was concentrated in the endothelial cells of the reticular zone (fig. 4 *D*), in contrast to the other dye-proteins, which were found in the medulla. In addition, the adjacent epithelial cells of the reticular zone contained a sprinkling of fine amber granules. The lymph nodes contained much less dye-protein than was observed in those of the animals of the previous series, while the tissue macrophages in various parts of the body only occasionally

10. (*a*) Maximow, A. A., and Bloom, W.: A Textbook of Histology, ed. 3, Philadelphia, W. B. Saunders Company, 1938. (*b*) Cowdry, E. V.: A Textbook of Histology: Functional Significance of Cells and Intercellular Substances, ed. 2, Philadelphia, Lea & Febiger, 1938, p. 436. (*c*) Evans, H. M., and Scott, K.: In Carnegie Institution of Washington: Contributions to Embryology, Publication 10, 1921.

contained the dye. The mitral valve of the heart, however, contained as much as in the case of the EB-HA and OG-B-HA animals (fig. 5 *A*). Three animals showed Anitschkow cells or "myocytes" containing the dye.

In the kidney the contrast with the previous series was striking. All the glomeruli contained many scattered amber granules, while in the glomeruli of the animals killed at one day there were heavy focal accumulations (fig. 5 *B*). These heavy foci could possibly represent circulating macrophages which became lodged in the glomerular capillaries. To distinguish the cytoplasm of endothelial cells from that of epithelial cells in the glomeruli, periodic acid and modified Lee-Brown stains for basement membranes were made. Although most of the acriflavine-colored granules were found in the endothelial cells, a few granules were found in the cytoplasm of the epithelial cells.

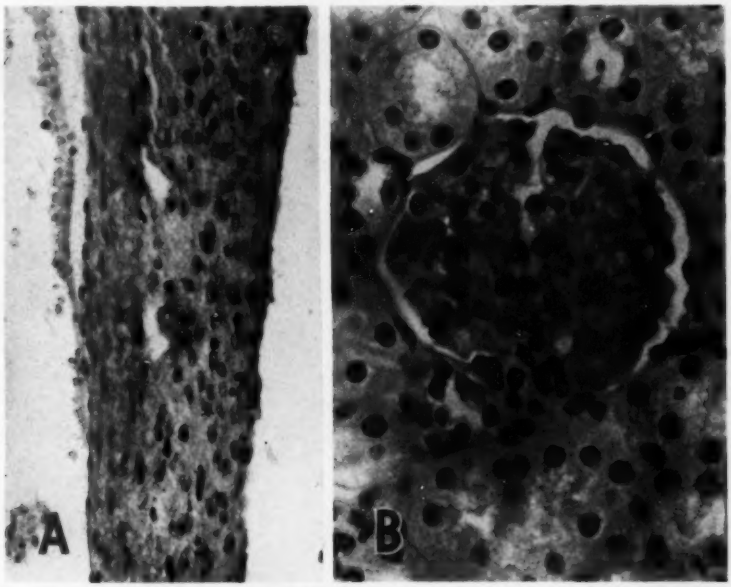

Fig. 5.—*A*, mitral valve, acriflavine-azo-human serum albumin (A-HA), rabbit 7, after eight days; × 520. The dark granules are in the atrial layer.

B, kidney, A-HA, rabbit 2, after one day; × 520. The granules are present only in the glomerulus. Arrows mark larger amber masses, possibly contained in circulating macrophages that have lodged in a capillary. The cells of the tubules are free of amber granules. (Compare with figs. 3 *A* and 6 *B*).

Acriflavine Alone.—No dye was found microscopically in any of the tissues of two rabbits killed one day after injection of acriflavine alone.

India Ink.—Because most of the A-HA appeared in the cells of the spleen, the bone marrow and the liver, which are in close contact with the circulating blood, while relatively little found its way into the connective tissue macrophages, it seemed of interest to compare its behavior with that of a particulate substance. It was found that 6 cc. of centrifuged Higgins' waterproof india ink could be injected without killing the animal. The animals were put to death the following day.

In addition to the usual microscopic observations,[10a] certain details are of interest here. The atrial surface of the mitral valve showed areas of macrophages laden with particles (fig. 6 *A*).

Within the right ventricle fibrinous clots contained carbon. Many macrophages throughout the lung parenchyma contained carbon particles, but almost none were found around the larger bronchi or vessels. In the kidney carbon particles were scattered through every glomerulus but were completely absent from the tubules (fig. 6*B*). Many macrophages between the tubules also contained carbon. In the glomerulus the scattered carbon particles were definitely demonstrated to be associated with endothelial cells by the periodic acid basement membrane stains. The stains demonstrated no thickening of the basement membrane except for one animal with early glomerular nephritis. As with A-HA, focal clumps were also seen that could possibly represent circulating macrophages lodged in a glomerular capillary. Only portions of the lymph nodes had carbon in the littoral cells and adjacent macrophages. Numerous small carbon particles were scattered through the cytoplasm of the liver cells themselves. The distribution is quite similar to that seen following injection of acriflavine-azo-human serum albumin (table).

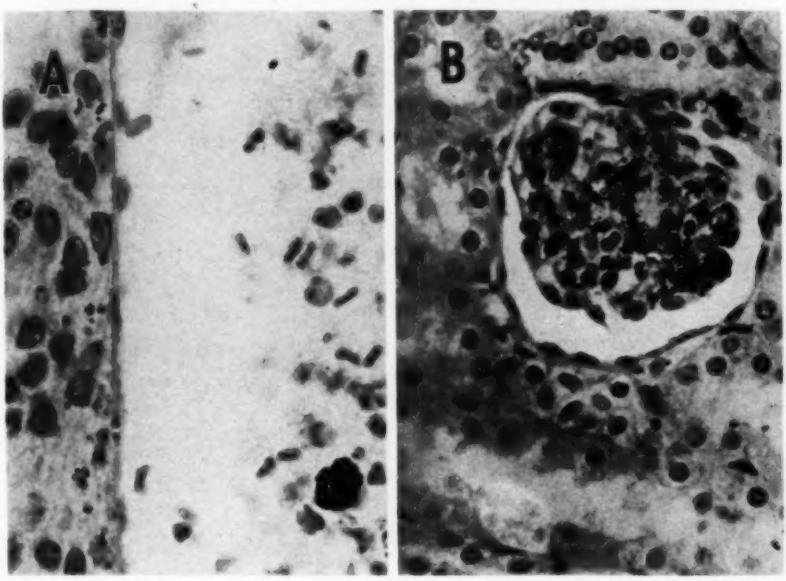

Fig. 6.—*A*, mitral valve, india ink, rabbit 105, after one day; × 780. The ink particles are in the atrial layer of the valve. Among the erythrocytes in the chamber is a circulating macrophage heavily laden with ink particles.

B, kidney, india ink, rabbit 105, after one day; × 520. Large numbers of ink particles are lodged in the glomerulus, while none are found in the cells of the tubules.

COMMENT

Stability of the Azoprotein Molecule.—In regard to the localization of foreign protein the significance of the results depends on the stability of the azoproteins in the animal body. Evidence for the relative chemical and immunologic stability of such dye-proteins has been presented by Heidelberger and Kendall [3a] and Landsteiner.[3c] Since the azo dyes alone are not antigenic, the development of antibody to these azo components after injection of the azoproteins suggests their relative stability until the antibody-forming mechanism of the body is reached. Unfor-

tunately, the cells responsible for this mechanism are unknown; evidence has favored variously macrophages, lymphocytes or lymphoblasts, or plasma cells.[11] Since extremely small quantities of antigen may stimulate antibody formation, the location of the greatest portion of a foreign protein does not necessarily implicate that site in antibody formation. In electrophoretic experiments on mixtures of dye-proteins, it has been shown that the individual proteins maintain their respective mobilities and colors without separation or recombination.[12] Different proteins labeled with the same dye appear in different sites when injected into mice, instead of in the same sites, as would be expected if the dye were first separated from the proteins.[5e] The control animals were given injections of comparable quantities of the dyes alone in order that the distribution of the dyes might be determined if the dye-protein complexes should be split in the body before decolorization took place. None of the dyes was diazotized before injection, for, as the method of preparation of the azoproteins indicates, the diazo substances combine readily with proteins in vitro, and Smetana [13] has assumed that in vivo they combine with the plasma proteins and has demonstrated their subsequent tissue localization.

As no dye was found in the microscopic preparations of the control rabbits which had received an injection of either acriflavine or orange G, it may be concluded that the amber granules appearing in the tissues of animals given A-HA and the red granules in the tissues of animals given OG-B-HA were conditioned by the human serum albumin in the body.

Evans blue, in contrast, was found in the microscopic sections even after it had been injected in the unconjugated form. Except for the kidneys and the circulating macrophages, dye granules were found only in rare tissue macrophages in these control animals, in contrast to the EB-HA animals. Evans blue has been shown to combine with serum albumin,[14] and it seems likely that its presence in these rabbits was due to its having combined with the animals' own plasma albumin. Evans blue, when injected repeatedly as such, can remain grossly visible in testis, adrenal, liver, kidney, periaortic tissue, teeth and cartilage for as long as six months.[15] In large doses it may prove lethal. Even when attached to a foreign protein it seemed to cause a disturbance of the clotting mechanism in the present animals. Other dyes are not innocuous.[16] Thus, although no evidences of toxicity of the OG-B-HA and A-HA were noted, minor degrees of intoxication may have influenced the

11. Bjørneboe, M.; Gormsen, H., and Lundquist, F. B.: J. Immunol. **55**:121, 1947. Ehrich, W. E., and Harris, T. N.: Science **101**:28, 1943. Rich, A. R.: The Pathogenesis of Tuberculosis, Springfield, Ill., Charles C Thomas, Publisher, 1944, p. 412.

12. Smetana, H., and Johnson, F. R.: Am. J. Path. **18**:1029, 1942.

13. Smetana, H.: Am. J. Path. **23**:255, 1947.

14. Rawson, R. A.: Am. J. Physiol. **138**:708, 1943.

15. Hueper, W. C., and Ichniowski, C. T.: Toxicopathologic Studies on Dye T-1824, Arch. Surg. **48**:17 (Jan.) 1944.

16. (*a*) Jaffé, R. H.: Physiol. Rev. **11**:277, 1931. (*b*) Kiyono, K.: Die vitale Karminspeicherung: Ein Beitrag zur Lehre von der vitalen Färbung mit besonder Berücksichtigung der Zelldifferenzierungen im entzündeten Gewebe, Jena, Gustav Fischer, 1914. (*c*) Kiyono, K.; Sugiyama, S., and Amano, S.: Acta scholae med. univ. imp. in Kyoto. **21**:1, 1938.

results of these experiments. It may be concluded that after the injection of EB-HA most of the blue granules in tissues other than the kidney were conditioned by the human serum albumin.

Mechanisms.—Reasons for the differing behavior of human serum albumin when labeled with different azo dyes are obscure. Gitlin [5e] was unable to explain similar differences with other azoproteins in mice on the basis of their isoelectric points. The influence of net molecular charge may be important in the present experiments, since Evans blue and orange G which localized in a similar fashion have a negative charge in solution when combined with human albumin,[17] while acriflavine, which localized quite differently, has a positive charge in solution when combined.[17] The india ink particles, handled in the body like A-HA, also have a positive charge in solution, as determined by electrophoresis. The importance of the charge of dyes for the penetration of membranes is discussed by Höber.[18]

Molecular or particle size may be a factor, as had been shown in the case of heterologous serum proteins [9a] and chemically related but relatively inert macromolecular substances.[19] Large particles leave the blood stream in a matter of seconds, smaller ones in minutes.[20] India ink and graphite suspensions are removed in times varying from a few minutes to a few hours.[21] Bacteria are removed in minutes, and more rapidly in immune animals [22] unless a more virulent culture produces septicemia.[23] Soluble dyes may remain over 24 hours.[24] In contrast almost all the animals given an injection of a dye-protein in the present experiments still had a measurable concentration in the circulating blood seven to 10 days later. Preliminary ultracentrifugal studies on these azoproteins, however, have revealed slight differences in the range of particle sizes in each solution [5] but not sufficient to account for the differences encountered in tissue localization.

The idea has been expressed [5e] that large doses might saturate one cell type and then force into activity or visibility some other cell types or tissues. Such has been the experience with vital dyes [25] and with iodine.[26] Even with the tremendous doses given here, the lymphocytes and lymphoblasts remained free of visible dye-protein, and only rarely did macrophages at the edge of the peripheral zone of lymphocytes in follicles show colored granules. Since extremely small

17. Conn, H. J.: Biological Stains: A Handbook on the Nature and Uses of the Dyes Employed in the Biological Laboratory, ed. 5, Geneva, N. Y., Biotech. Publications, 1946.

18. Höber, R.: Physical Chemistry of Cells and Tissues, Philadelphia, The Blakiston Company, 1945.

19. Hueper, W. C.: Macromolecular Substances as Pathogenic Agents, Arch. Path. **33**: 267 (Feb.) 1942; Arteriosclerosis, ibid. **39**:117 (Feb.) 1945.

20. Dobson, E. L.; Gofman, J. W.; Jones, H. B.; Kelly, L. S., and Walker, L. A.: J. Lab. & Clin. Med. **34**:305, 1949. Gersh, I.: Anat. Rec. **70**:331, 1938.

21. (*a*) Drinker, C. K., and Churchill, E. D.: Proc. Roy. Soc., s.B **101**:462, 1927. (*b*) Jaffé.[16a] (*c*) Kiyono.[16b]

22. Cannon, P. R.; Sullivan, F. L., and Neckermann, E. F.: J. Exper. Med. **55**:121, 1932.

23. Enders, J. F.; Shaffer, M. F., and Wu, C.: J. Exper. Med. **64**:307, 1936.

24. Gregersen, M. I., and Rawson, R. A.: Am. J. Physiol. **138**:698, 1943.

25. (*a*) Aschoff, L.: Ergebn. d. inn. Med. u. Kinderh. **26**:1, 1924; (*b*) Lectures on Pathology, New York, Paul B. Hoeber, Inc., 1924.

26. Leblond, C. P.: In Lawrence, J. H., and Hamilton, J. G.: Advances in Biological and Medical Physics, New York, Academic Press, Inc., 1948, p. 353.

amounts of a foreign protein suffice for the production of antibody, it is possible that the dye-proteins could reach the lymphocytes and stimulate antibody formation in amounts too small to be visible. Harris and Ehrich [27] have recently found a soluble antigenic factor in an extract of lymph nodes after the injection of a particulate antigen which they regard as further evidence in favor of the theory that macrophages may merely predigest or prepare the antigen for the lymphocytes which make the antibodies.

It may be concluded that visualization of the azoproteins depends on the protein component, but the amount seen in any particular tissue is governed by the particular dye attached. Different mechanisms can be suggested to explain these differences. First, the primary localization of the different azoproteins may differ, or, second, the azoproteins may find their way into the involved cells in any particular location in equal quantities but be broken down or decolorized at different rates. Third, the dye may be separated from the protein elsewhere in the body and its local visualization depend on the common action of the foreign protein and the specific action of the free dye. While the evidence is not decisive in favor of any one mechanism, the present results certainly demonstrate a vast difference in activity of the cells in any given site depending on the particular prosthetic azo dye group attached to the protein.

Macrophage System.—The present observations lend emphasis to the role of the macrophage system [25] of the body, the macrophages taking up with ease a majority of substances from large particles to soluble dyes with relatively small molecular weights. Others [28] have previously noted the appearance of azoproteins in macrophages, but the distribution is studied in more detail here. There is evidence from the use of labeled antibodies that antigens accumulate in Kupffer cells,[5a] endothelial cells [5a] and macrophages.[5c] In a detailed study of the tissue localization of pneumococcic polysaccharides types 2 and 3, using fluorescent antibody, these carbohydrates were identified in the cytoplasm of lymphocytes.[29] The present experiments do not indicate how long after injection of the azoproteins colored granules may remain visible in the macrophages, for granules were seen in all the animals. Although the visible distribution of A-HA was quite different from that of EB-HA and OG-B-HA, the rates at which they disappeared from the circulation were quite similar,[8] indicating that about the same relative amount was removed in any given time. Because less A-HA appeared in the tissue macrophages or lymph nodes, it might be supposed that the blood vessels were less permeable to A-HA. However, the results could also be explained by assuming that the different azoproteins reached all the cells concerned in the same relative amounts but were decolorized at different rates.

Similarities Between Tissue Localization of Azoproteins and Sites of Hypersensitivity Lesions.—Heart: The constant localization of all the dye proteins in the cells of the cardiac valves was quite striking. There are several finer points that

27. Harris, T. N., and Ehrich, W. E.: J. Exper. Med. **84**:157, 1946.

28. Gitlin,[5c] Sabin,[31] Smetana,[13] Smetana and Johnson.[12]

29. Coons,[5a] Kaplan, M. H.; Coons, A. H., and Deane, H. W.: J. Exper. Med. **91**:15, 1950.

deserve comparison with experimental hypersensitivity cardiac lesions [30] as well as with rheumatic lesions in human hearts. Macrophages and cells resembling fibroblasts, both containing the colored granules, were found in the same layer of the mitral valve (figs. 1 *D*, 3 *D* and 5 *A*) in which are found human rheumatic lesions as well as the experimental lesions described by Rich and Gregory.[31] The ventricular surface of the aortic valve is a common site of rheumatic lesions, and, similarly, macrophages filled with colored granules were found in the plate of loose connective tissue there. Furthermore, tissues at the base of the valves as well as the chordae tendineae contained large numbers of granule-filled macrophages (fig. 2 *A*), and frequently these sites show lesions in rheumatic fever and in experimental serum disease. The myocardium, as well as the endocardium, may be involved here (fig. 2 *B* and *D*), in humans and in sensitized rabbits. In the hearts of the experimental animals showing myocarditis and pericarditis, the number of granule-containing macrophages was more pronounced in the diseased areas. As myocarditis was a frequent finding in both the experimental and the control groups, no relationship to the experimental procedure can be construed. The rabbits may well have been killed too early to show hypersensitivity lesions.

It would be of some interest to discover whether the dye-proteins reached their site in the valves by diffusing directly from the chambers of the heart or from vessels in the valves themselves. This is the same problem that arises in the consideration of the pathogenesis of bacterial endocarditis.[32]

As noted above, A-HA, which was not found nearly as much as EB-HA in tissue macrophages, did find its way into the valves in amounts comparable to EB-HA. That large particles could penetrate into the valvular tissues was shown by the india ink that localized there (fig. 6 *A*).

Differentiating macrophages from fibroblasts is difficult, especially in fixed tissue sections. It has long been known that both can contain dye granules but that macrophages show them more readily.[33] However, fibroblasts can retain their dye bodies six months or more, while macrophages lose theirs much more rapidly.[10c] While the morphological criteria given are helpful, the best method of differentiation lies in studying the living cells with supravital dyes.

There is no difficulty, on the other hand, in identifying an Anitschkow cell or "myocyte," which plays a prominent part in the myocarditis of experimental hypersensitivity and the myocarditis of rheumatic fever and of other diseases. A number of Anitschkow cells containing azoprotein granules were found in these animals. Dye granules have previously been observed in these cells.[34]

Kidney: The finding of dye granules in the epithelial cells of renal tubules is an old observation.[16b] Smetana and Johnson [12] and Smetana [13] demonstrated that

30. (*a*) Hawn and Janeway.[9a] (*b*) More and McLean.[9b] (*c*) More, R. H.; Waugh, D., and Kobernick, S. D.: J. Exper. Med. **89**:555, 1949. (*d*) Murphy, G. E., and Swift, H. F.: Ibid. **89**:687, 1949. (*e*) Rich, A. R., and Gregory, J. E.: Bull. Johns Hopkins Hosp. **73**:239, 1943; (*f*) **75**:115, 1944.

31. Rich and Gregory, footnotes 9 c, 30 e, 30 f.

32. Forbus, W. D.: Reaction to Injury: Pathology for Students of Disease Based on the Functional and Morphological Responses of Tissues to Injurious Agents, Baltimore, Williams & Wilkins Company, 1943, p. 425.

33. Aschoff.[25a] Aschoff.[25b] Evans and Scott.[10c] Kiyono.[16b] Maximow.[10a]

34. Zak, F. G.: Anat. Rec. **98**:25, 1947.

various azoproteins (including a serum globulin) could filter through the mammalian glomerulus and be reabsorbed by the lining cells of the proximal convoluted tubules, with the formation of colloid droplets. Gitlin [5e] extended these observations, finding that by diazotizing a different dye to the same protein he could alter its behavior in the kidney. The unexpected localization of granules in all glomeruli of A-HA-treated animals, with no trace of this dye-protein in the tubules, is quite interesting. Smetana observed granules of dye-protein in phagocytes in glomeruli as we did in rabbits of the present series killed at one day but not in the groups killed later. The regular presence of granules in the glomeruli of animals killed after a longer interval must be due to a different mechanism. It is tempting to consider this in the light of the experimental work indicating that glomerulonephritis may be due to an antigen-antibody reaction.[35] One of the experimental rabbits did, in fact, show an early glomerulonephritis, but the finding of a more advanced stage in another rabbit not given an injection of a dye or a dye-protein suggests that the former may have had a naturally occurring renal disease.

The observation that india ink particles can lodge in the glomerular endothelium (confirming Smetana [5o]) suggests that particle size may be a factor, yet Bremer [36] found trypan blue granules in both the endothelial and the epithelial cells of rat glomeruli.

Lungs: The fact that the dye-proteins were regularly localized in the macrophages around the pulmonary arteries of the rabbits suggests comparison with the facts that rabbits dying in anaphylactic shock have marked constriction of the pulmonary arteries and that lesions of the pulmonary arteries have been found in hypersensitive rabbits.[9b] The concentration of macrophages just outside the smooth muscle of the bronchi is strikingly similar to the localization of antigen in guinea pigs killed in anaphylactic shock.[5p]

The irregular distribution of the dye in macrophages of the lung parenchyma may be compared with the variable presence and focal character of human rheumatic pneumonitis and experimental anaphylactic pneumonitis.[37]

The presence in the lung parenchyma of cells containing amber granules after injection of A-HA might possibly be explained if macrophages in the circulation had lodged in the pulmonary capillaries. This mechanism appears to operate following the injection of dyes.[38] It would not, however, explain the presence of blue granules in cells of the alveolar tissue of EB-HA-treated rabbits.

Synovial Membrane: The synovial tissues deserve special consideration because of the frequency with which they are involved in rheumatic fever and rheumatoid

35. Ahlström, C. G.: Acta path. et microbiol. Scandinav. 1936, supp. 29, p. 1. Cavelti, P. A., and Cavelti, E. S.: Studies on Pathogenesis of Glomerulonephritis: Clinical and Pathologic Aspects of Experimental Glomerulonephritis Produced in Rats by Means of Autoantibodies to Kidneys, Arch. Path. **40**:163 (Sept.) 1945. Ehrich, E. W.; Seifter, J., and Forman, C.: J. Exper. Med. **89**:23, 1949. Hawn and Janeway.[9a] Kay, C. F.: J. Exper. Med. **72**:559, 1940. Leiter, L.: Ann. Int. Med. **28**:229, 1948. Schwentker, F. F., and Comploier, F. C.: J. Exper. Med. **70**:223, 1939. Smadel and Swift.[5a] Solomon, D. H.; Gardella, J. W.; Fanger, H.; Dethier, F. M., and Ferrebee, J. W.: J. Exper. Med. **90**:267, 1949.

36. Bremer, J. L.: Anat. Rec. **70**:263, 1938.

37. Gregory, J. E., and Rich, A. R.: Bull. Johns Hopkins Hosp. **78**:1, 1946.

38. Maximow, A. A., in Cowdry, E. V.: Special Cytology: The Form and Functions of the Cell in Health and Disease, ed. 2, New York, Paul B. Hoeber, Inc., 1932, p. 425.

arthritis and because of the lesions found in hypersensitive animals.[39] In the animals that received injections of azoproteins the cells of the synovial membranes regularly contained colored granules. Only occasional cells containing india ink particles were found. The localizing of trypan blue (but not india ink) in the superficial part of the synovial tissues, especially following irritation or trauma, has been described as occurring after the dye had been injected into the abdominal wall.[40] In the experiments just mentioned and in the present ones, cells resembling fibrocytes were seen to contain colored granules. In fact, the synovial cells themselves can take up foreign substances, as has been observed with hemosiderin in hemarthroses.[41]

Connective Tissue and Arteries: When present in tissue macrophages, all the azoproteins were seen in those parts of most active movement, for example: esophagus, synovial membrane, intestine, skeletal muscle and more specifically near the arteries in these tissues.

The tendency of the particles to occur in cells singly or in groups in the adventitia of arteries of myocardium, lung, renal pelvis, skeletal muscle and pancreas suggests the possibility that such localization is correlated with the variable occurrence of the focal lesions of periarteritis nodosa, both in experimental animals and in man.[9]

Liver: The finding of the specific granules and even of ink particles in liver cells is unexplained. The same observation has been made previously with dye and carbon particles.[42] None of the substances injected in the present experiments was seen in the bile passages. These observations may have some bearing on the focal necrosis of liver cells which has been described in experimental hypersensitivity. Buckley and associates [43] concluded from tissue culture experiments that liver cells from sensitized rabbits were not damaged by contact with specific antibody but that focal hepatic necrosis probably resulted from injury to the endothelial cells lining the hepatic sinusoids. These endothelial cells concentrate antigens to a much greater degree than the liver cells, as the present experiments demonstrate.

SUMMARY

In rabbits, after intravenous injection of three different soluble azoproteins (Evans blue-azo-human serum albumin, orange G-azo-benzidine-azo-human serum albumin, and acriflavine-azo-human serum albumin), correspondingly colored granules were vizualized predominantly in the macrophage system throughout the body. Granules were also found in Anitschkow "myocytes" and in the epithelial cells of the liver, the adrenal cortex and the proximal convoluted tubules of the

39. Hawn and Janeway.[9a] More and McLean.[9b]

40. Kuhns, J. G., and Weatherford, H. L.: Role of Reticulo-Endothelial System in Deposition of Colloidal and Particulate Matter in Articular Cavities, Arch. Surg. **33**:68 (July) 1936.

41. Bennett, G. A.: in Anderson, W. A. D.: Pathology, St. Louis, C. V. Mosby Company, 1948, p. 1,327.

42. Kiyono.[16b] Maximow.[16a]

43. Buckley, J. J.; Buckley, S. M., and Gey, M. K.: Bull. Johns Hopkins Hosp. **84**:195, 1949.

kidney. In addition, fibroblasts and synovial cells appeared to contain granules. These results are in significant contrast with those observed on similarly prepared tissues after a single injection of the dyes alone.

No granules were found associated with lymphocytes or lymphoblasts, in spite of tremendous doses of injected azoproteins.

Notable differences in the distribution of dye granules depend on the particular azo dye attached to the protein.

More fundamental interpretations of the results will depend on an understanding of the mechanisms by which the azoproteins are handled in the body. Possible mechanisms and the relative stability of the azoproteins in vivo are discussed.

In many tissues, specific colored granules were found in the cells in highest concentration in those sites where lesions are known to develop in hypersensitive animals and in human beings with diseases strongly suspected of having a basis in anaphylactic hypersensitivity.

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THE EARLY LESIONS OF CANINE ARTERIOSCLEROSIS

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EARLIER work from this laboratory showed that long-sustained hypercholesteremia in dogs resulted in the production of arterial lesions strikingly similar both in distribution and in morphology to those seen in human arteriosclerosis.[1]

Studies have now been made on the natural history of the development of the lesions. In the original investigations dogs were maintained on a cholesterol-thiouracil regimen for 14 months. This period was arbitrarily chosen, and no information was obtained on either the length of time and the degree of hypercholesteremia required to produce the earliest lesions or on the sequence of events that take place in the walls of the arteries as the lesions develop. This report deals with observations on 30 dogs killed after being maintained on the cholesterol-thiouracil regimen for one to six months.

MATERIALS AND METHODS

Mongrel dogs of known parentage were placed on experiment at the age of 4 months. They were housed on a farm in groups of not more than six, with free access to outdoor exercise yards and fed ad libitum a diet containing 5 per cent cholesterol. Each dog received 0.6 Gm. of thiouracil daily by mouth. These experimental conditions seem more favorable than those used in the earlier experiments, in which the dogs were confined in laboratory cages and offered definite amounts of the cholesterol-containing food. In both experiments the diet, which was moistened with milk immediately before feeding, consisted of "Spratt's kibbled dog cakes® #34," containing 5 per cent added cholesterol.

The ration is prepared by dissolving cholesterol in ether, mixing this with the dog food and allowing the ether to evaporate. The cholesterol is distributed throughout the food in finely divided form. The cholesterol-treated ration keeps well, and large quantities can be prepared at one time and stored.

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From the Research Service, First (Columbia University) Division, Goldwater Memorial Hospital, Department of Hospitals, and the Departments of Medicine and Pathology, College of Physicians and Surgeons, Columbia University.

1. (*a*) Steiner, A., and Kendall, F. E.: Atherosclerosis and Arteriosclerosis in Dogs Following Ingestion of Cholesterol and Thiouracil, Arch. Path. **42**:433-444 (Oct.) 1946, (*b*) Steiner, A.; Kendall, F. E., and Bevans, M.: Production of Arteriosclerosis in Dogs by Cholesterol and Thiouracil Feeding, Am. Heart J. **38**:34-42, 1949. (*c*) Combined Staff Clinics: Cholesterol Metabolism and Arteriosclerosis, Am. J. Med. **6**:110-113, 1949.

Analysis of the feces indicates that from 60 to 80 per cent of the cholesterol fed in this way is absorbed. Only 20 per cent is absorbed when the cholesterol is fed in coarsely crystalline form.[1c]

Blood samples were taken for cholesterol analysis at intervals of two weeks. Determinations were made by the Schoenheimer-Sperry method.[2]

The animals were killed either by excessive pentobarbital sodium narcosis or by a combination of narcosis and exsanguination. Examination included dissection of the entire arterial system to the smallest branches possible and gross and microscopic examination of the viscera as well as of the arteries. Routine sections were stained with hematoxylin and eosin and special stains as indicated. Frozen sections were stained for lipid using oil red O according to the method of Lillie.[3] All the arterial lesions were tabulated and graded by a single observer. The degree of involvement of the various arteries was estimated on a scale of 1 to 4+, in which 1+ indicates the presence of a few scattered pinpoint lesions and 4+ almost complete involvement of the intima of the vessel.

RESULTS

Serum Cholesterol Levels.—Serum cholesterol levels rose rapidly, reaching 1,000 mg. per 100 cc. within one month in the majority of the animals. The degree of hypercholesteremia reached and maintained depended on the amount of cholesterol consumed. To maintain high levels the dogs must be in perfect health, have plenty of exercise and be eating well. Hot weather, worms, mange, fleas and even loneliness have all been observed to result in lower cholesterol levels. Figure 1 shows that the serum level of cholesterol will fall from 1,500 mg. per 100 cc. to a normal value of 200 mg. per 100 cc. within a week of withdrawing cholesterol and thiouracil from the diet. This dependence of the serum level on cholesterol intake probably explains the large variations in serum cholesterol level shown in figures 2, 3 and 4, where the levels measured at intervals of two weeks are represented.

Relation of Gross Lesions to Serum Cholesterol Levels.—One dog was examined after the serum cholesterol had been maintained at an average level of 1,260 mg. per 100 cc. for one month. No gross lesions were found. Gross lesions have been found in all of four dogs with serum levels maintained over 1,200 mg. per 100 cc. for two months and in all dogs with levels over 700 mg. per 100 cc. for four or six months. In the four-month group there were 17 dogs; in the six-month group, eight dogs. Figures 2, 3 and 4 show the biweekly cholesterol levels and the distribution and relative intensity of the lesions found in representative animals examined two, four and six months after the cholesterol-thiouracil regimen was initiated. The vessels most frequently involved are listed in the graphs, which are incomplete in that occasionally vessels not listed will show greater involvement than those shown. As might be expected, individual dogs show great variation in the distribution and intensity of the lesions even though comparable serum cholesterol levels are maintained. However there is fair correlation between the average cholesterol level and the total involvement, a value obtained by adding the values assigned to indicate the intensity of the lesions found in the eight sites listed in the graphs. Figure 5 shows the data for 14 dogs examined after four months on the regimen. The lesions in the eight dogs killed after six months on the regimen were larger and more widely distributed than in the two previous groups despite lower average serum cholesterol levels.

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2. Schoenheimer, R., and Sperry, W. M.: A Micro-Method for the Determination of Free and Combined Cholesterol, J. Biol. Chem. **106**:745-760, 1934.

3. Lillie, R. D.: Histopathologic Technic, Philadelphia, The Blakiston Company, 1948, p. 158.

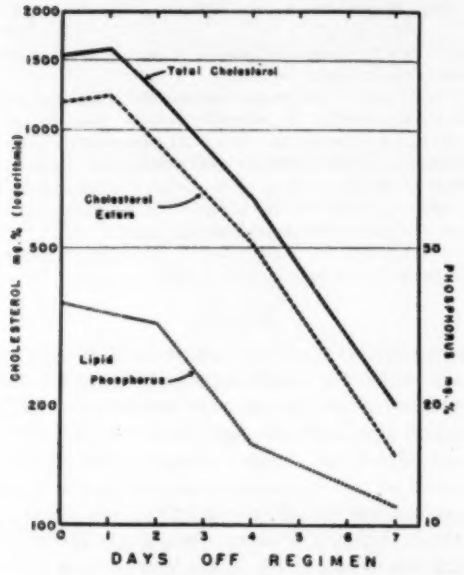

Fig. 1.—An illustration of the rapidity with which total serum cholesterol, cholesterol esters and lipid phosphorus fall to normal levels in a dog taken off the thiouracil-cholesterol regimen for one week.

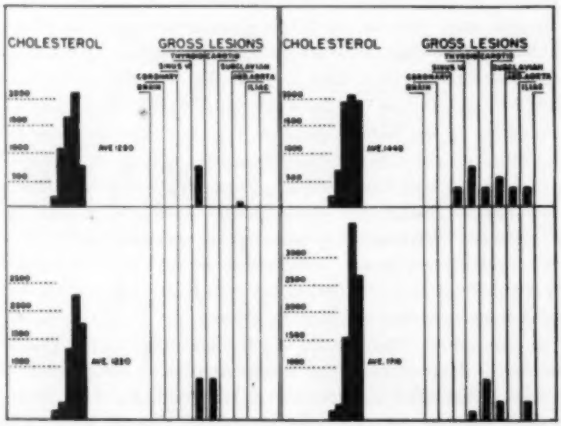

Fig. 2.—Biweekly and average serum cholesterol levels in 4 dogs maintained on the thiouracil-cholesterol regimen for two months. These are correlated with the gross lesions of arteriosclerosis graded from 1 to 4+ (see text).

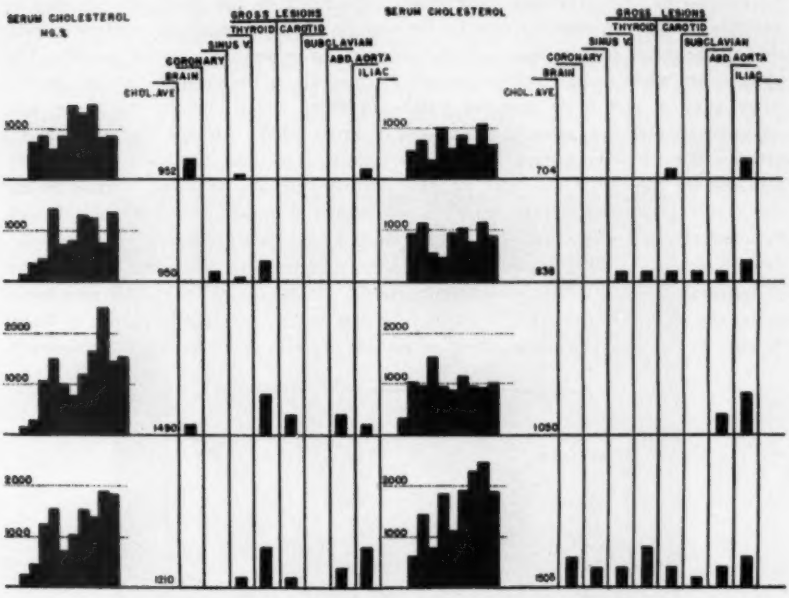

Fig. 3.—Same schema as in figure 2—for eight dogs on the regimen for four months.

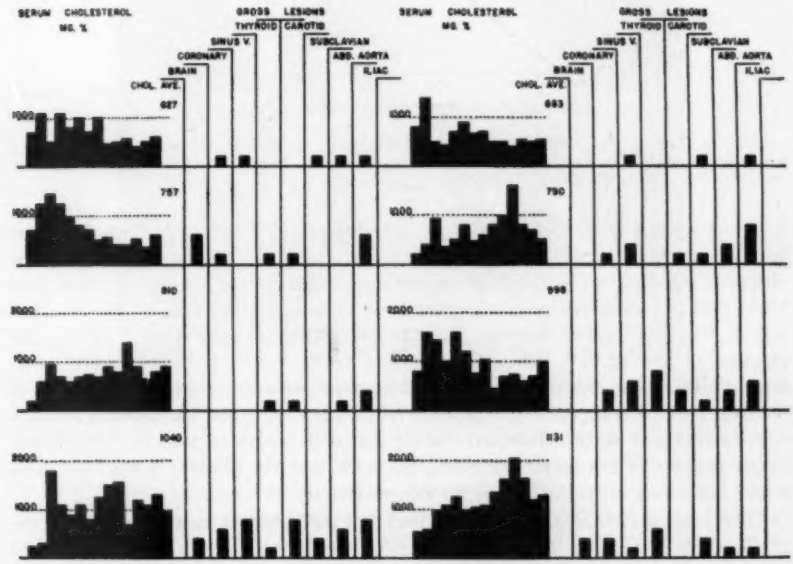

Fig. 4.—Same schema as in figures 2 and 3—for eight dogs on the regimen for six months.

Pathologic Observations.—The gross appearance of the early plaques seen in animals on the regimen for two to six months is not significantly different from that of the more mature lesions found in dogs on experiment for 14 months. The plaques are white or pale yellow and are elevated above the intimal surface (fig. 6). They vary in size from pinpoint lesions to those which encircle the vessel and obviously reduce the lumen of the vessels of small caliber. In the iliac and femoral arteries these lesions assume an annular pattern, since they are most often superimposed on the extra muscular bundles normally present in these arteries in the dog. To a certain extent this pattern is also observed in the carotid arteries, though here the ridges are less well defined and do not encircle the artery.

Individual variation among animals makes it difficult to determine the sequence of appearance of the lesions in different arteries. However, the thyroid artery seems to be the first one involved. This may be due to the increased volume of blood flowing to the gland in consequence of the hyperplasia produced by the thiouracil.

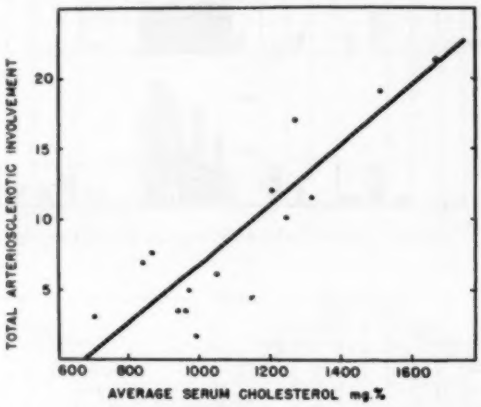

Fig. 5.—The total number of plus signs as illustrated in figures 2, 3 and 4 plotted against the average cholesterol values obtained for the serums of 14 dogs on the regimen for four months. The rough degree of correlation is apparent.

There is a tendency for lesions to appear in arteries of small caliber which supply muscles before the larger vessels are severely involved. Such early lesions have been observed in branches of the femoral, cervical, thoracic and lumbar arteries. The lower part of the abdominal aorta at the bifurcation and at the exits of the branches was a frequent site of lesions. Longitudinal streaks comparable to the "milk plaques" of infants were occasionally present in this location. Plaques were sometimes found at the exit of the celiac and superior mesenteric arteries. Lesions in the iliac, femoral and popliteal arteries developed gradually and increased in severity with increases of serum cholesterol levels and with length of time on experiment. Gross plaques in the ascending aorta, the arch and the thoracic aorta have been found but rarely in animals kept on experiment up to six months.

The sinus of Valsalva, the anterior leaflet of the mitral valve and the base of the aortic valve were sites of early predilection in the heart. Lesions of the small subepicardial branches of the coronary arteries were seen earlier and more fre-

quently than were plaques in the major branches. Cerebral lesions have been found only in animals which had extensive lesions elsewhere. They occurred principally in areas of sharp angulation and branching in the vessels of the circle of Willis.

Histologically the early plaque differed from the mature lesion. At *two months* all the elevation of the plaque was due to the spreading apart of the medial fibers by large amounts of lipid, which was also present within the fibrocytes and smooth

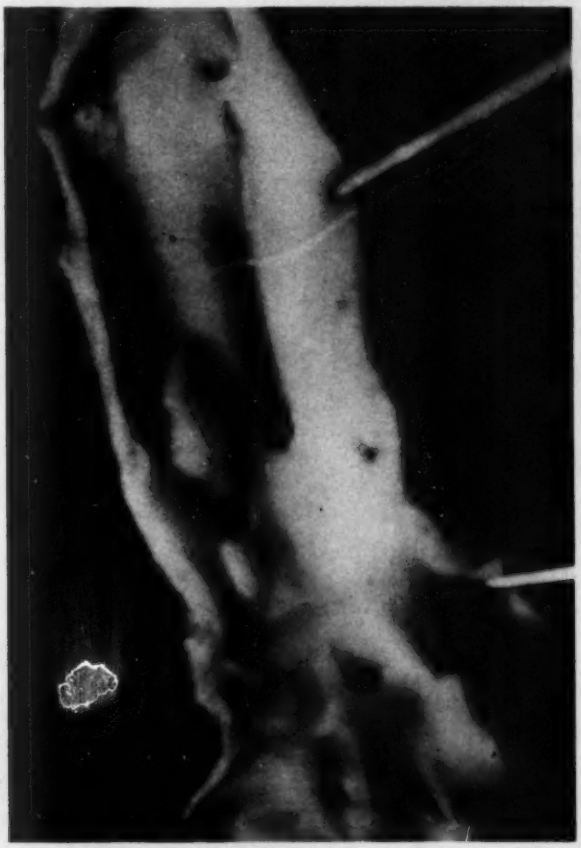

Fig. 6.—Plaques in the abdominal aorta of a dog on the regimen for six months; × 3.

muscle cells (figs. 7 and 8 *A*). The endothelial cells comprising the entire intima of arteries, including the aorta, in these young puppies were swollen and could be stained by sudan dyes. The internal elastica was similarly swollen and stainable but remained intact. At *four months* intimal proliferation occurred sporadically and when present was minimal. In animals killed after *six months* the intimal proliferation was consistently present in addition to large amounts of lipid in the media (fig. 8 *B*). The internal elastica at this time was often fragmented and reduplicated.

A few foam cells were present in the intima but not in the media in these lesions. Ulceration, hemorrhage and calcification were not seen in these early plaques, in contrast to the plaques of dogs on experiment for 14 months.

Fig. 7.—Thyroid artery of a dog on the regimen for two months; frozen section stained with oil red O; × 23. Lipid deposition is marked by light areas in the media. Intimal proliferation is absent.

The livers often appeared large and pale and on section were friable and greasy. The spleens were not remarkable save for occasional plaques in the small arteries entering the hilus. Some of the kidneys had pale yellow streaks in the cortex. Microscopically, the parenchymal cells of the liver, as well as the Kupffer cells and

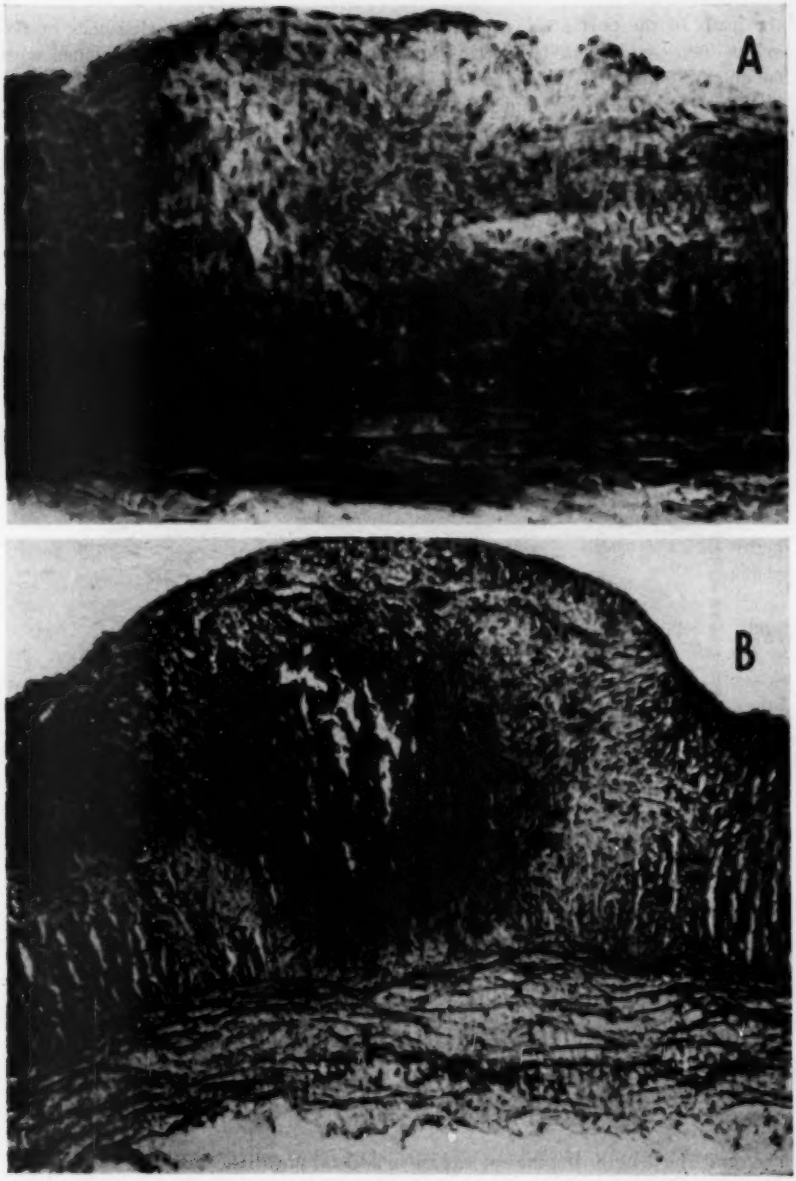

Fig. 8.—*A*, carotid artery; frozen section stained with oil red O; × 153. The section was made through a plaque in a dog on the regimen for two months. Lipid is indicated by pale areas.

B, iliac artery; frozen section stained with oil red O; × 153. The section was made through a plaque in a dog on experiment for six months. Sudanophilic areas appear dark. Note the intimal proliferation.

the walls of the portal veins, contained lipid. The reticuloendothelial cells of the spleen were swollen, and the walls of the arteries and arterioles were laden with sudanophilic material. In the kidney, lipid was present in the capillary endothelium in the ascending loops of Henle and the collecting tubules. The arterioles and arteries contained varying amounts of lipid. In general the height of the serum cholesterol

Fig. 9.—Plaque from a child aged 12 years; frozen section stained with oil red O; × 180. It shows minimal intimal proliferation. Lipid infiltration of the inner layers of the media is indicated by dark areas.

level and the length of time on experiment determined the intensity of the fatty change.

The thyroid gland was uniformly hyperplastic, with many intravesicular papillary infoldings. The lining epithelium was tall. The colloid content varied but was greatly reduced. When present, it was most prominent in the periphery of the gland. With fat stains, lipid was found in the perivascular connective tissue as well as in the walls of the arteries.

COMMENT

We have intentionally used young dogs to avoid the possibility of spontaneous arteriosclerosis, which is known to occur in elderly animals.[4] Whereas in the experiments on mature dogs, previously reported, the lesions were strikingly similar to those of human disease, these early lesions differ in microscopic appearance but not in distribution. Again, their extent, as in the older dogs, is roughly a function of the time on experiment and the elevation of the serum cholesterol level.

The use of young dogs introduced a factor which undoubtedly influences the morphologic aspects of the lesions. In puppies, as in human infants, the intima of the aorta consists only of endothelium resting on the internal elastica. By about the tenth month a fibrous layer develops beneath the intima in the dog. The lack of intimal proliferation in the plaque before *four months* and the consistency of its appearance in *six months* (when our animals reach the age of 10 months) may well be a reflection of this anatomic feature. It is conceivable that with the constant washing of heavily lipemic serum through the wall any increase in the thickness of the intimal layer impedes the free flow and thus disturbs the rather precarious colloidal dispersion of the plasma lipids. Then their prolonged presence within the intima may stimulate a proliferative reaction. Undoubtedly, the same factors which operate to localize the plaque in human arteriosclerosis are active in the experimental disease in dogs, i. e., interruption of the continuity of the vessel lumen, irregularities of the intimal surface and increased hydrostatic pressure. The role, if any, which injury to the endothelial cell plays in the localization of lesions is not clarified by these studies.

The early lesions are characterized by lipid accumulating in the media about the vasa vasorum as well as in the endothelial cells. Some of this lipid must be transported directly to the media by the vasa, but part of it certainly seeps through the intact endothelium. This feature can be traced from the incipiency of the lesion, when only the intima and the inner two or three layers of the media (where vasa vasorum do not exist) are sudanophilic.

The lesions which occur in young children and infants bear a rather close resemblance to these early canine lesions if the difference in the concentration of the serum lipids is appreciated. Obviously there are few infants with serum cholesterol levels sustained at 1,000 mg. per 100 cc. for any length of time. Nevertheless, these juvenile plaques are often seen without marked intimal proliferation and with infiltration of inner layers of the media (fig. 9).

SUMMARY AND CONCLUSIONS

A series of puppies 4 months of age were placed on a regimen known to produce arteriosclerosis. They were killed at intervals from one to six months. The earliest gross lesions appeared after two months of sustained hypercholesteremia and were similar to those observed in dogs which had been on experiment for 14 months. Histologically they differed in that intimal proliferation was rare until the sixth month, most of the elevation of the plaque being due to intramedial accumulation of lipid. There was a rough correlation between the degree of the hypercholesteremia, the length of time on experiment and the extent of the lesions produced.

4. Cowdry, E. V.: Arteriosclerosis: A Survey of the Problem, New York, The Macmillan Company, 1933, p. 159, p. 276. Zinserling, W. D.: Vergleichend-morphologische Untersuchungen über Atherosklerose: Über bindegewebige Intimaverdickungen und spontane Lipoidose der Aorta und anderer Organe bei Hunden, Beitr. z. path. Anat. u. z. allg. Path. **88**:241-314, 1932.

REGRESSION OF LESIONS IN CANINE ARTERIOSCLEROSIS

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THE EXPERIMENTAL arteriosclerosis produced in dogs [1] under conditions which permit controlled observations has made it possible to investigate several problems of great practical interest in relation to the management of arteriosclerosis in man.

The question of whether arteriosclerotic lesions regress when the inciting cause is removed or whether the lesion once initiated inevitably goes on to maturity is of fundamental importance. The obvious difficulty of observing changes in lesions in man has resulted in more speculation than information on this subject.

Although the canine arteriosclerosis is produced under conditions different from those which are active in the development of the human disease, in their morphologic aspects and distribution the lesions are strikingly similar. It seems probable that once arteriosclerotic lesions have formed in dogs they will be influenced by the same factors that operate in human beings. Studies of changes which occur in these lesions after the dogs are taken off the arteriosclerosis-producing regimen of cholesterol-thiouracil feeding and after their serum cholesterol levels have become normal should yield pertinent information concerning these factors. Knowledge of the changes which take place spontaneously is required for an evaluation of the effect of any therapeutic measure on the lesion.

MATERIAL AND METHODS

Four dogs were maintained for four months on the cholesterol-thiouracil regimen described previously.[1a] They were then fed a diet of "Spratt's kibbled dog cakes® # 34" without added cholesterol or thiouracil. Two of these dogs were examined after two months and two after four months on the stock diet. Likewise nine dogs were maintained for six months with high serum cholesterol levels and examined four months after being taken off the regimen. The

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From the Research Service, First (Columbia University) Division, Goldwater Memorial Hospital, Department of Hospitals, and the Departments of Medicine and Pathology, College of Physicians and Surgeons, Columbia University.

1. (*a*) Combined Staff Clinics: Cholesterol Metabolism and Arteriosclerosis, Am. J. Med. **6**:110-113, 1949. (*b*) Steiner, A., and Kendall, F. E.: Atherosclerosis and Arteriosclerosis in Dogs Following Ingestion of Cholesterol and Thiouracil, Arch. Path. **42**:433-444 (Oct.) 1946. (*c*) Steiner, A.; Kendall, F. E., and Bevans, M.: Production of Arteriosclerosis in Dogs by Cholesterol and Thiouracil Feeding, Am. Heart J. **38**:34-42, 1949.

TABLE 1.—*Extent and Intensity of Arteriosclerosis in Dogs on a Thiouracil-Cholesterol Regimen*

Dog	Av. Serum Cholesterol, Mg. per 100 Cc.	Sclerotic Lesions of Arteries							
		Cerebral	Coronary	Valsalva Sinus	Thyroid	Carotid	Subclavian	Abd. Aorta	Iliac
Dogs 4 Months on Regimen, Examined Immediately									
41	704	−	−	−	−	+	−	−	++
40	840	−	−	+	+	+	+	+	++
36	870	−	+	±	++++	−	+	±	+
30	940	−	+	±	++	−	−	−	−
43	950	++	−	±	−	−	−	−	+
33	970	−	+	−	++	−	−	+	+
35	990	−	−	−	−	−	−	+	+
6	1,050	−	−	−	−	−	−	++	++++
70	1,110	+	++	+	+	+	−	+	++
29	1,190	+	−	−	++++	++	−	++	+
10	1,210	−	−	+	++++	++	−	++	+
71	1,220	+	+	+	+	+	−	−	++
37	1,250	−	−	+	++++	+	−	++	++++
34	1,310	+	++++	++	+++	++	−	+++	++
42	1,490	+++	++	++	++++	++	+	++	+++
38	1,670	++++	++++	++	++++	+	+	+++	++
73	1,866	+	++	++++	+++	++++	+	+++	++++
Dogs 4 Months on Regimen, Examined 2 Months Later									
1	1,210	−	−	−	−	+	+	+	+++
20	1,320	−	±	+	++	+	−	±	+
Dogs 4 Months on Regimen, Examined 4 Months Later									
18	1,100	−	±	±	+	++	−	+	++
19	1,430	−	±	−	+	+	±	−	+

Key: The significance of the minus and plus signs is the same as in the preceding article in this issue of the Archives.

TABLE 2.—*Extent and Intensity of Arteriosclerosis in Dogs on Thiouracil-Cholesterol Regimen*

Dog	Av. Serum Cholesterol, Mg. per 100 Cc.	Sclerotic Lesions of Arteries							
		Cerebral	Coronary	Valsalva Sinus	Thyroid	Carotid	Subclavian	Abd. Aorta	Iliac
Dogs 6 Months on Regimen, Examined Immediately									
44	630	−	+	+	−	−	+	+	+
45	680	−	−	+	−	−	+	−	+
32	760	+++	+	−	+	+	−	−	+++
3	790	−	+	++	−	+	+	++	++++
4	810	−	−	−	+	+	−	+	++
31	1,000	−	++	+++	++++	++	+	++	+++
16	1,040	++	+++	++++	+	++++	++	+++	++++
494	1,130	++	++	+	+++	−	++	+	+
Dogs 6 Months on Regimen, Examined 4 Months Later									
47	460	−	−	−	−	−	−	−	+
48	640	−	−	−	−	−	−	−	+
54	700	−	−	−	−	−	−	−	+
56	750	+	−	−	−	−	−	−	+
53	850	−	−	−	−	−	−	−	++
13	980	++	+	±	+	+	+	+	++
52	1,230	+	−	+	+	+	−	−	+
14	1,500	+	++	−	++	+	+	++	+
55	1,830	+	+	+	++	++	+	+++	++

controls for this experiment are discussed in a previous paper in which 14 dogs were examined immediately after four months and eight dogs after six months on the cholesterol-thiouracil regimen.[2] The methods used for determining the serum cholesterol levels and for evaluating the degree of arteriosclerosis present were identical with those described in that paper.

RESULTS

During the cholesterol-thiouracil feeding period the serum cholesterol levels showed fluctuations similar to those previously reported for the control dogs.[2] The levels fell to normal within a week after the feeding of the dogs was changed to the stock diet and remained down throughout the rest of the experiment. The

Fig. 1.—Iliac artery of a dog kept on a cholesterol-thiouracil regimen for four months and then on the stock diet for two months. Scarcely any lipid can be detected in the intima and the upper media. The only appreciable amount remaining is about a vasa vasorum in the outer media and in the adventitia. This area has been blocked off. Frozen section; oil red O stains; × 175.

average serum cholesterol level for each dog during the cholesterol-thiouracil period is given in tables 1 and 2, together with the extent of the lesions found on gross examination in the eight sites most commonly affected. These observations when compared with those of the control dogs clearly showed that the lesions diminished in both number and intensity during the period on the stock diet, when the serum

2. Bevans, M.; Davidson, J. D., and Abell, L. L.: The Early Lesions of Canine Arteriosclerosis, Arch. Path., this issue, p. 278.

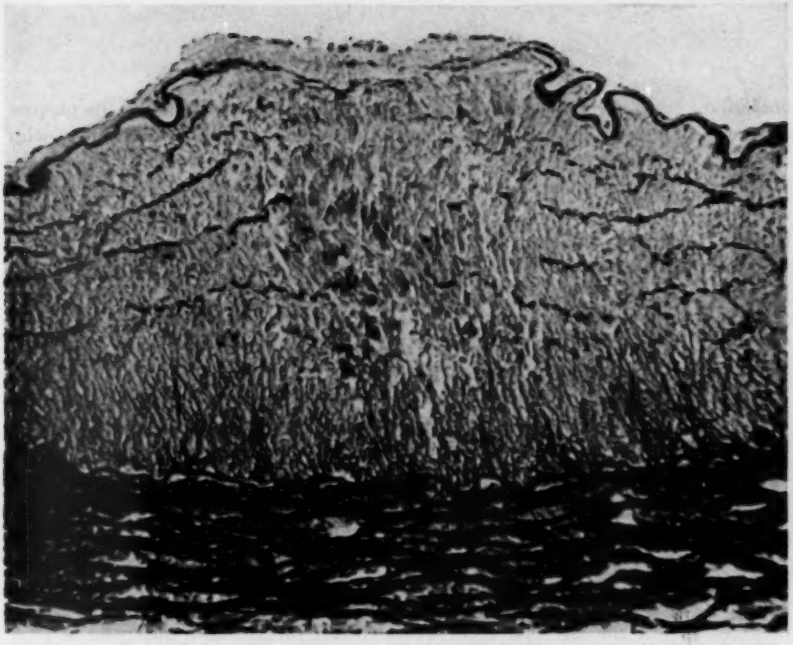

Fig. 2.—Iliac artery from a dog kept on a thiouracil-cholesterol regimen for four months and then on the stock diet for four months before being killed. Note the slightly thickened intima and medial scarring. No lipid could be stained in this lesion. Elastic tissue Van Gieson stain; × 167.5.

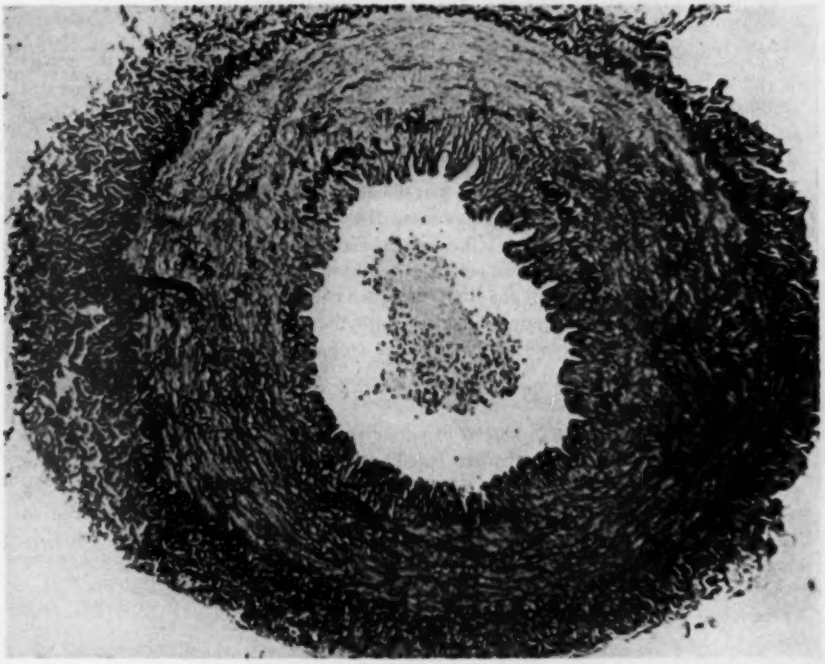

Fig. 3.—Section taken through a grossly normal portion of the thyroid artery of a dog which showed sustained hypercholesteremia for 15 months before it was killed. Note disruption of elastic fibrils. Elastic tissue Van Gieson stain; × 80.

cholesterol levels were normal. Furthermore, the gross appearance of the plaques differed in that they were less conspicuous. The lesions tended to be flatter, with indefinite borders that blended imperceptibly into the normal intima.

Microscopic examination of sections of many of these lesions stained to visualize fat showed that the slightly thickened intima contained little or no lipid. Small flecks of lipid were still detectable in the inner media, and relatively large amounts were present in the outer media and in the adventitia directly beneath (fig. 1). In general, the longer the animal had been off the experimental regimen, the more the amounts of lipid were decreased. These aggregations tended to accumulate about the vasa vasorum of both the outer media and the adventitia, suggesting their removal, presumably via lymphatic channels.

Sections were taken through areas of arteries which appeared normal grossly. Microscopically, some of these arteries showed a slightly thickened intima, which contained no demonstrable fat. Beneath, the media was scarred and the elastic fibers were distorted, but no lipid was found except for occasional flecks in the outer media (fig. 2). These areas were interpreted as sites of preexisting plaques which had regressed after the cholesterol-thiouracil regimen was interrupted.

In studies of dogs on the arteriosclerosis-producing regimen for longer periods, i. e., up to 14 months, the lesions in the thyroid arteries often appeared less extensive and less severe than those in animals on the regimen up to six months. This occurred despite the fact that their serum cholesterol had been sustained at high levels. Sections of the thyroid arteries of these animals through grossly normal areas showed scarring of the media with minimal intimal thickening and no stainable lipid (fig. 3). This evidence is highly suggestive that plaques may disappear even in the presence of long-sustained hypercholesteremia.

COMMENT

Lipid is rapidly removed from both the intima and the media, and the repair of the wall is probably complete in many of the early lesions, since only traces of residual lesions are seen four months after cessation of the cholesterol-thiouracil regimen.

The observations on the thyroid arteries have further interest in that these arteries are probably unique from a physiological point of view in these animals. During the first few months on experiment the dogs' thyroid glands are enlarging and becoming hyperplastic under the influence of thiouracil. This puts an added demand on the thyroid arteries such as does not occur in any other arteries of the body. It seems probable that the high incidence of arteriosclerosis in these arteries is related to their hypertrophy coincident with that of the glands and that in the later months this developmental process has stopped with the cessation of gland enlargement.

CONCLUSIONS

Arteriosclerotic lesions produced in young dogs kept on a cholesterol-thiouracil regimen for four to six months are capable of regression and probably ultimate disappearance when the dogs are taken off the regimen and fed a stock diet for two to four months before being killed. Serum cholesterol levels are restored to baselines within one week. Regression of the plaques despite sustained hypercholesteremia has also been observed, particularly in the thyroid arteries.

THE TESTIS

III. Absence of Germ Cells; Sclerosing Tubular Degeneration; "Male Climacteric"

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PART I of this study dealt with the normal anatomy of the testis,[1] and part II described the histopathological appearance of the testes of a group of apparently healthy men in whom the only abnormal clinical finding was oligospermia or azoospermia.[2] Various disorders of spermatogenesis or, if spermatogenesis was normal, atresia of the excretory ducts usually accounted for the deficiency of sperm in the semen. No endocrine imbalance was detected in these men.

The present paper deals with a group of patients with testicular disease and demonstrable endocrine imbalance manifested by increased excretion of pituitary gonadotropins and sometimes a diminished output of 17-ketosteroids with or without signs of eunuchoidism. The primary defect was believed to be in the testis, for reasons that will be discussed. The clinical and laboratory studies included a careful evaluation of the endocrine status, estimations of the pituitary gonadotropins and the 17-ketosteroids excreted in the urine, semen analyses and one or more testicular biopsies. The observations on 55 men are reported.

The patients could be separated into three distinct categories on the basis of the histological abnormalities observed in the testis: (1) those with no germ cells in the seminiferous tubules but without other definite abnormalities of the gland; (2) those showing progressive tubular sclerosis and hyperplasia of the Leydig cells; (3) those without obvious abnormalities of the testis as judged from routine preparations, but with definite imbalance of the gonadotropic hormones.

The clinical details of the study have been recorded in another publication.[3] The case numbers given in the earlier reports have been retained here.

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Dr. Howard was R. P. Campbell Memorial Fellow, Montreal General Hospital, 1947. Present Address: Montreal General Hospital, Montreal, Que., Canada.

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2. Sniffen, R. C.; Howard, R. P., and Simmons, F. A.: The Testis: II. Abnormalities of Spermatogenesis; Atresia of the Excretory Ducts, Arch. Path. **50**:285 (Sept.) 1950.

3. Howard, R. P.; Sniffen, R. C.; Simmons, F. A., and Albright, F.: J. Clin. Endocrinol. **10**:121, 1950.

ABSENCE OF GERM CELLS

On the basis of histology this group of 19 patients falls into logical sequence with the men reported in part II of this study,[2] who showed severe hypospermatogenesis. It is a short step from severe hypospermatogenesis to aspermatogenesis, but certain historical, physical and endocrinological aberrations may be found in men with the latter condition. The physical and endocrine normality of the patients with hypospermatogenesis and arrest of spermatogenesis has been stressed.[4]

With one exception, the men in the present group were married, potent but infertile, and normally developed. Case 96 concerned an unmarried man of 18

TABLE 1.—*Absence of Germ Cells*

Case	Age, Yr.	Size of Testes	17-Ketosteroids (Urinary), Mg./Day*	Follicle-Stimulating Hormone (Urinary), M. U./Day†		Sperm Counts§
78	35	Small	6.2	+52	—104 (D)‡	0
79	36	Normal	5.9	+13	—52 (D)	0
				+192		
80	30	Small	6.4	+13	—104 (D)	0
				+96		
81	28	Small	11.3	+13	—104 (D)	
				+96	—192	2/hpf
82	24	Small	11.9	+13	—52 (D)	0
				+48	—96	5 M./cc.
83	29	Small	3.0	+13	—104 (D)	0
				+192		
84	33	Small	9.2	+13	—104 (D)	0
				+96		
85	38	Small	5.6	+104 D		
				+96	—192	0
86	28	Small	5.2	+52 D		
				+96	—192	0
87	31	Small	10.5	+104 D		
					—104 (D)	0
88	30	Small	8.8	+96	—192	0
89	26	Normal	8.7	+13	—104 (D)	0
90	25	Normal	9.0	+13	—52 (D)	0
				+96	—192	
91	26	Small	9.4	+13	—104 (D)	0
92	27	Normal	11.7	+26	—52 (D)	0
				+192	—384	
93	32	Small	14.0		—208	2/hpf
				+104 D		2/hpf
94	31	Small	11.2	+104 D		2 M./cc.
						5/hpf
95	22	Small	5.2	+13	—52 (D)	0
96	18	Normal	10.2		—96	..
				+13 D		

* The levels of urinary 17-ketosteroids were determined by a modification of the Zimmermann reaction. The range for normal men lies between 8 and 18 mg. per day. Fraser, R. W.; Forbes, A. P.; Albright, F.; Sulkowitch, H., and Reifenstein, E. C., Jr.: J. Clin. Endocrinol. 1: 234, 1941.

† The urinary follicle-stimulating hormone was determined by the method of H. F. Klinefelter Jr., F. Albright and C. C. Griswold (J. Clin Endocrinol. 3:529, 1943). By "the dialysis method" the normal range for positive responses is 6.5 to 26 mouse units per day; positive values at 52 m. u. or above are abnormally high. By "the nondialysis method," which is designed to differentiate between normal and high levels of follicle-stimulating hormone, positive responses of 10 m. u. per 100 cc. or of 96 m. u. per day are abnormally high.

‡ D indicates values obtained by the dialysis method; other results were obtained by the nondialysis method.

§ A search for sperm and sperm counts were made several times in each case. M./cc. = millions per cubic centimeter; hpf = high power field.

years with large breasts. Some of the pertinent data relating to these patients are indicated in table 1. Several points deserve mention. First, the testes were usually moderately reduced in size. Second, the urinary 17-ketosteroids were sometimes low. Third, the excretion of follicle-stimulating hormone tended to be high, and

4. Sniffen and others.[2] Howard and others.[3]

in three cases was greatly elevated. Finally, 14 patients had no sperm in their semen on repeated examinations. It is evident, therefore, that the condition often entails a degree of endocrine imbalance that is not evident in the physical development of the individual.

Microscopically, the testes of these men showed a moderate decrease in the diameter of the tubules, and with two exceptions no spermatogenic cells were present. The cell population of the tubules was composed entirely of mature Sertoli cells, which seemed to be flourishing and were distinctly outlined (fig. 1). Often the individual cells could be distinguished from one another by a well defined cytoplasmic membrane in the basal portion of the cell. They were tall cells that lay vertical to the basement membrane, and sometimes their apexes were driven to one side like windswept treetops. As in normal sustentacular cells, the cytoplasm contained fine and coarse granules with wavy "fibrils" traversing the long axis.

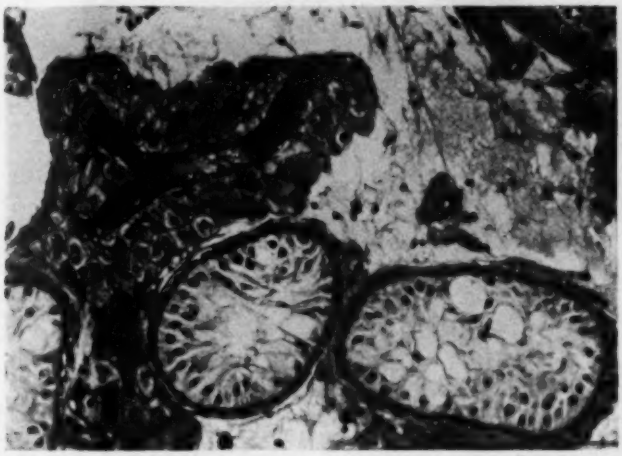

Fig. 1.—Absence of germ cells. The diameter of the tubules is decreased, but there is no thickening of the tunica propria. No germ cells are present, and the tubules are lined by normal Sertoli cells containing large lipid droplets in the cytoplasm. A large group of normal Leydig cells envelops a branching capillary. Phloxine-methylene blue stain; × 200.

Usually crystalloids were present, and in three cases they were extraordinarily numerous. The nuclei varied from round to oval, had a varying chromatin content and contained prominent plasmosomes. They lay at various levels in the cytoplasm, and the sharply outlined nuclear membrane often lacked the characteristic wrinkling found in the normal gland. Sometimes an unusual number of fat droplets were present in the cytoplasm and often these were encountered in the lumen as foam. Occasionally the lumens were empty or were filled in by the tips of the Sertoli cells. The lumens of other tubules contained desquamated Sertoli cells in a good state of preservation. These cells had retained their structure, including the crystalloids, but stained with great intensity. The basement membrane of the tubule was never thickened, but in slightly more than half the patients the tunica propria was slightly or moderately thickened by a deposition of collagen and elastic fibrils.

This abnormality was not striking and never approached the degree of sclerosis found in certain other conditions.

Two specimens taken for biopsies (cases 82 and 96) each showed a single active tubule or at least a segment of a tubule. In one instance the activity was limited to spermatogonia and spermatocytes; in the other, sperm were being formed. Table 1 indicates that three other men had a few sperm in the semen; so presumably the biopsies missed active tubules. This fact would seem to be highly significant in a consideration of the genesis of the lesion.

The interstitial tissue of the testes was not always normal. In 15 patients the number of Leydig cells appeared to be within the normal range, but in the other four there was a slight to moderate numerical increase in the cells. The decrease in tubular diameter incident to aspermatogenesis with a consequent drop in testicular volume did not seem to explain adequately the increase in the Leydig cell

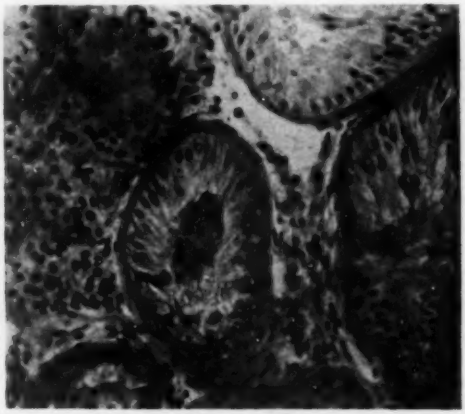

Fig. 2.—Absence of germ cells. The interstitial tissue contains many groups of "spindle" cells. Phosphotungstic acid-hematoxylin stain; × 200.

mass; the cells were concentrated at various points in the interstitial tissue in medium-sized or large groups or they filled in the intertubular spaces almost entirely.

The appearance of the individual Leydig cells was normal in all the glands except two, and in these a number of the cells were swollen and the cytoplasm was filled completely with fine droplets. Disintegrating cells were not unusually numerous, and no appreciable alteration had occurred in the formation of secretory droplets, crystalloids and pigment. In a few cases all the Leydig cells seemed to be in a high degree of secretory activity and were large, with many droplets in the periphery of the cytoplasm; thus well defined "ectoplasmic" and "endoplasmic" zones had developed (fig. 1).

In seven cases there was an abnormal increase in the number of cells resembling fibrocytes in the interstitial tissue. These cells had a tendency to proliferate in groups, and because of their vague outline and the character of the nuclei they were often difficult to distinguish from Leydig cells (fig. 2). Some of these "spindle" cells may be capable of developing into Leydig cells. In any event their presence is abnormal and may indicate a hormonal imbalance.

SCLEROSING TUBULAR DEGENERATION

In 1942 Klinefelter, Reifenstein and Albright[5] published their observations on nine men with a syndrome characterized by "bilateral gynecomastia, small testes, aspermatogenesis, evidence of normal to moderately reduced function of the Leydig

TABLE 2.—*Sclerosing Tubular Degeneration*

Group	Case	Age, Yr.	Gynecomastia¶	17-Ketosteroids (Urinary), Mg./Day*	Follicle-Stimulating Hormone (Urinary), M. U./Day†
(a) With little "eunuchoidism" but with gynecomastia	97	30	+++	13.1	+205 −328
	98	21	+++	7.2	+150 −240
	99	31	+++	10.3	+257 −385 +192
	100	37	+	11.7	+384 −192
	101	25	++	3.4	+13 −104 (D) +192 −384
	102	17	+++	7.2	+30 −50/100 cc. +220 −370
	103	15	++	5.1	+192 −384 +96 −384
	104	38	++	8.8	+104 (D) +192
	105	24	+++	14.7	+13 −26 (D) +104 (D)
	106 (later)	34	0 +	13.3	+26 −52 (D) +104 (D)
	107	31	++	16.3	+10/100 cc. +384
	108	26	+++	10.1	+96 −192 +96 −192
	109	42	++	9.0	+192
	110	32	+++	9.6	+10/100 cc. +270 −450
	111	37	+++	8.6	+261 −391 +192
(b) With little "eunuchoidism" and without gynecomastia	112	32	0	N. D.	+10/100 cc.
	113	19	0	4.9	+192
	114	29	0	12.0	+13 −104 (D) +384
	115	32	0	9.1	−10/100 cc. +192 later
	116	30	0	N. D.	+10/100 cc.
(c) With marked "eunuchoidism" and with gynecomastia	117	45	+	4.7	+104 (D) +192
	118	17	+++	1.8?	+100/100 cc. +612 −979
	119	18	+++	4.6	+30 −100/100 cc. +50 −100/100 cc.
	120	38	+	6.3	+104 (D) +384
	121	31	+	6.6	+192 +192
	122	19	+++	4.0	+576 +384 −576
(d) With marked "eunuchoidism" and without gynecomastia	123	22	0	7.0	+50 −100/100 cc. +96 −192
	124	29	0	3.5	+52 −104 (D) +96 −192
(e) Doubtful examples	125	14	+++	0.9	+104 (D)
	126	17	+++	7.0	+108 −212

* For key, see footnote to table 1.
† For key, see footnote to table 1.
¶ Gynecomastia is described as ranging from 0 (absent) to +++ (marked).

cells, increased excretion of follicle-stimulating hormone, and usually a reduced excretion of 17-ketosteroids." Since that time it has been found that there is great

5. Klinefelter, H. F., Jr.; Reifenstein, E. C., Jr., and Albright, F.: J. Clin. Endocrinol. **2**:615, 1942.

variability in the features of the syndrome. However, two abnormalities in the condition remain constant, namely, an elevation of the urinary gonadotropins and progressive degenerative changes in the testicle. The present report is an outline of the histopathological changes in the testes of 30 men with increased excretion of pituitary gonadotropins and includes seven of the patients originally studied by Klinefelter, Reifenstein and Albright.

The pertinent clinical and laboratory findings are shown in table 2. All the patients showed elevated urinary gonadotropins. The men have been grouped into five categories on clinical grounds. The first four categories were based on the severity of the various stigmas of eunuchoidism together with the presence or the absence of gynecomastia. The last group included two young men with high excretion of follicle-stimulating hormone and gynecomastia who may be in the early stages of the disease. The 17-ketosteroid excretion was decidedly variable in the 20 men showing little eunuchoidism with or without gynecomastia. However, in these persons the average excretion level was appreciably higher than the average level in the group showing marked eunuchoidism with or without gynecomastia. In a general way, therefore, some correlation seemed to exist between the clinical status of the patients and the 17-ketosteroid excretion. No correlation was evident between the excretion of follicle-stimulating hormone and the 17-ketosteroid level or the physical findings in the patients.

In general, in this syndrome the testes showed a progressive failure of spermatogenesis, accompanied by tubular sclerosis, and a marked increase in the number of Leydig cells per unit area. Many variations on this theme were encountered.

The cruder details of the process by which a normal seminiferous tubule was converted into a collagenous cord could be traced with ease in the testicular specimens from the 30 men of this group, and sometimes all the obvious changes were encountered in a single fragment of testis. The first abnormality noticed in the tubules was a dropping off of spermatogenic activity (fig. 3 *A*). This change did not affect all the tubules, nor was it a constant finding in different segments of the same tubule. Furthermore, even though spermatogonia were present in the usual numbers, spermatogenesis was frequently arrested after the development of primary spermatocytes. These events led to a decrease in the sperm population of the Sertoli cytoplasm, and unusually large numbers of primary spermatocytes had desquamated into the tubular lumens. Up to this point the findings followed the accustomed pattern of tubular failure. Hypospermatogenesis was followed closely by atrophy of the Sertoli cells without obvious changes in the nuclei of these elements, and gradually the tunica propria became thickened. Sometimes desquamated Sertoli cells were found in the lumens of the tubules. Further progression of the lesion was seen in an exaggeration of the abnormalities just enumerated. Consequently, tubules were encountered that showed merely a few spermatogonia lying in the shrunken Sertoli cytoplasm and a markedly thickened tunica propria. The germ cells were usually small with a relatively thin rim of dense cytoplasm, and cellular divisions were infrequent among them. Other tubules were lined merely by Sertoli cells (fig. 3 *B*). Finally, the few remaining germ cells and the Sertoli cells were wiped out, and the lumen of the tubule was obliterated by the thick, contracted tunica propria. In this way the tubules were converted into shrunken collagenous cords (fig. 4).

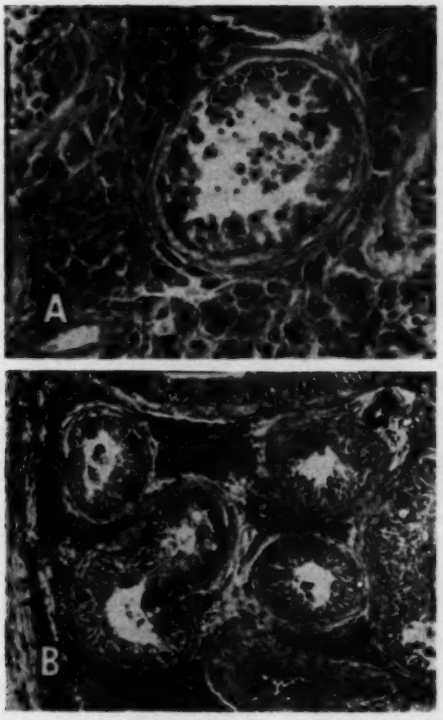

Fig. 3.—Sclerosing tubular degeneration. In *A* the tubule shows a decrease in spermatogenic activity. In addition there are thickening of the tunica propria and an increase in Leydig cells. In *B* a group of tubules may be seen, which lack spermatogenic cells and are lined by Sertoli cells that are beginning to atrophy. The tunica propria is thickened, and the Leydig cells are increased in number. Phloxine-methylene blue stain; × 200.

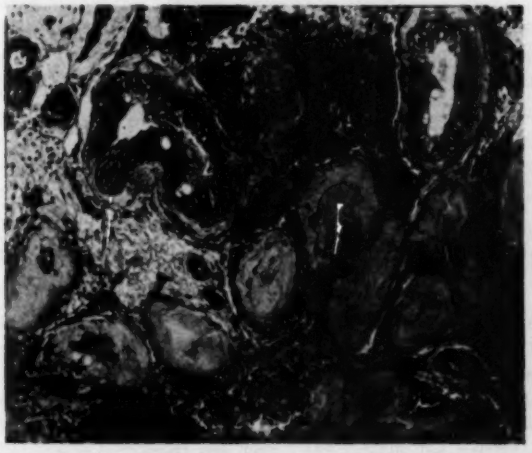

Fig. 4.—Sclerosing tubular degeneration. A group of sclerotic tubules have been converted into fibrous cords. Two tubular cross sections are not slerotic but lack germ cells and are lined by Sertoli cells that are beginning to atrophy. Phloxine-methylene blue stain; × 200.

Certain aspects of this process deserve emphasis. Atrophy of the sustentacular cells of the tubules was a feature of this condition and progressed by a gradual diminution of the cytoplasmic mass with the loss of "fibrils," crystalloids and lipoid vacuoles. Sertoli cell atrophy was always accompanied by thickening of the tunica, and the ratio between these two elements seemed to be in inverse proportion. A large number of the testes contained at least a few tubules that were lined by Sertoli cells which had not become atrophic, though they lacked germ cells. These sustentacular cells appeared to be healthy and contained cytoplasmic fibrils and crystalloids, and often a large number of lipoid droplets were present in the cytoplasm of the cell and in the lumen of the tubule. If atrophy of Sertoli cells had not occurred, there was little or no thickening of the tunica of the tubule, despite the failure of spermatogenesis.

In various reports on tubular sclerosis the process has been considered the result of thickening of the basement membrane.[6] This concept does not agree with our observations. We feel that the tunica propria is thickened by a deposition of fine, concentrically arranged collagen fibrils between its inner lamellas. This contention was supported by the fact that some of the nuclei in the tunica propria were carried inward as the deposition of collagen increased until finally the nuclei occupied a central position in the sclerotic tubule. During this entire process a delicate basement membrane could be identified lying between the cellular elements that remained in the tubule and the surrounding collagen and inwardly displaced cells of the tunica propria. The basement membrane was visualized most satisfactorily with silver stains and the periodic acid, basic leucofuchsin technic.[7] The membrane had disappeared by the time the tubule was totally sclerotic. Usually the deposited collagen was peppered with fine fuchsinophilic granules that did not have the tinctorial properties of collagen. The cells of the tunica propria that had been crowded centrally in the sclerosed tubules occasionally seemed to acquire cytoplasm and resembled Leydig cells. However, no crystalloids were ever found in their cytoplasm, and certainly such cells may be the remnants of atrophic Sertoli cells.

In contrast to the tubular sclerosis of senility [1] and that following damage to the hypophysis,[8] the outer layers of the tunica propria in most cases remained unchanged, nor had elastic fibrils been deposited in and around the lamellas as in the former conditions.

The clinical contention that the setting for this syndrome is laid during puberty [9] was supported by the microscopic observations of the testis. In 8 men undamaged immature tubules were scattered throughout the sections (fig. 5). These tubules were small, and each had an unaltered tunica propria and sometimes a lumen. No cell boundaries were discernible in the intratubular cytoplasm, which contained many dense, immature nuclei with or without visible plasmosomes. Occasionally

6. (*a*) Nelson, W. O., and Heller, C. G.: J. Clin. Endocrinol. **5**:13, 1945. (*b*) Engle, E. T.: J. Urol. **57**:789, 1947.

7. McManus, J. F. A.: Am. J. Path. **24**:643, 1948.

8. Sniffen, R. C.; Howard, R. P., and Simmons, F. A.: Arch. Path, to be published.

9. (*a*) Heller, C. G., and Nelson, W. O.: J. Clin. Endocrinol. **5**:1, 1945. (*b*) Howard and others.[5] (*c*) Kleinfelter and others.[5] (*d*) Nelson and Heller.[6a]

a few inactive spermatogonia were present, but no spermatocytes were found. The testes of two thirds of the patients contained small sclerotic cords that were thought to be the scarred remains of immature tubules. The appearance was not unlike the scars of atretic follicles as compared to corpora albicantia in the ovary. Probably these signs of tubular immaturity would have been found even more often if greater volumes of tissue had been available for section.

The interstitial cells were distinctly unusual in all the cases but one. The number of Leydig cells was increased per unit area in 23 of the 30 patients, and usually to an impressive degree. The number was believed to represent an absolute increase over and above the relative increment consequent to a decrease in the volume of the gland following tubular sclerosis.

In such conditions as "healed mumps orchitis," myotonia atrophica and the tubular sclerosis produced by roentgen rays and by impairment of the blood supply

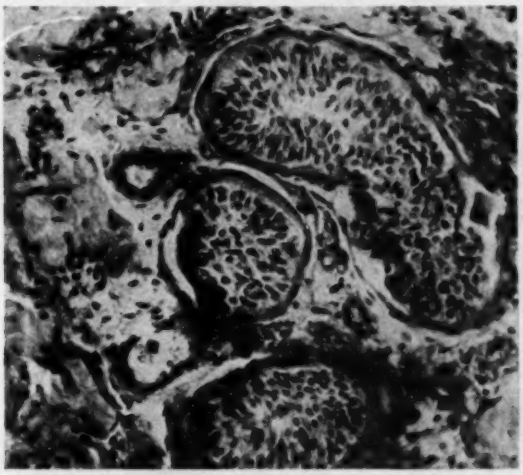

Fig. 5.—Sclerosing tubular degeneration. Cross sections of an immature tubule are seen. The cells lining the tubule are undifferentiated. The nuclei are more numerous than in a mature tubule; some nuclei are small, with compact chromatin, while many are larger, with visible plasmosomes. There is slight thickening of the tunica propria. No germ cells are present at the level of section. Phloxine-methylene blue stain; × 200.

of the testis, which may result in complete tubular sclerosis, the density of the Leydig cell population is far from comparable with the hyperplasia that occurred in the present syndrome. If the increase in interstitial cells were due to fusion of neighboring groups of cells one might expect condensation of the reticulum fibrils that always enclose each Leydig cell. No change in the reticulum pattern was evident in these testes, and therefore it was felt that the structure was more in keeping with hyperplasia than with condensation of the cells.

From the standpoint of morphology the testes of these patients could be divided into three categories depending on the degree of Leydig cell abnormality. Stress was laid on the appearance of the cells rather than on their numerical increase or grouping.

In nine cases the Leydig cell abnormality was impressive (fig. 6*A*). In these cases the interstitial tissue was punctuated by dense groups of slender fusiform cells resembling fibrocytes, but with small, round nuclei. These cells occasionally completely dominated the interstitial tissue and were sometimes associated with a slight increase of collagen fibrils. The recognizable Leydig cells lay in small or

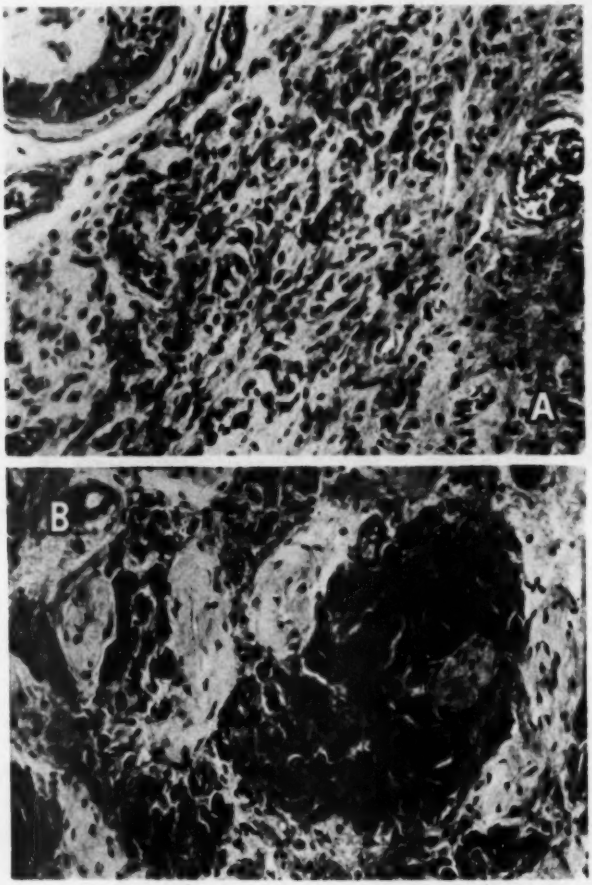

Fig. 6.—Sclerosing tubular degeneration. In *A* a group of predominantly spindle-shaped interstitial cells may be seen, showing marked variation in shape and size. These cells have ill defined cytoplasmic membranes and do not contain secretory droplets, crystalloids or pigment granules. Phloxine-methylene blue stain; × 200.

B shows a group of reasonably well-defined Leydig cells. In general, there is poor definition of the cytoplasmic membranes, the cells vary notably in size, many nuclei are pyknotic, and secretory droplets, crystalloids and pigment are not visible. Hematoxylin and eosin stain; × 200.

medium-sized groups and were not normal. They had a tendency to form short cords or to lie in masses in which the cell outlines were indistinct or indiscernible. The cytoplasmic mass of these cells was extremely variable in amount and was

often fusiform or distorted. The cytoplasm was finely granular, and large hyaline droplets were unusually numerous. In this group of cases the Leydig cells seldom contained secretory droplets, crystalloids or pigment. Nuclear pyknosis and anuclear cytoplasmic smudges were frequent. The arterioles of the interstitial tissue often seemed thick-walled. The tubules gave evidence of immaturity in each of the nine cases. In every biopsy, tubules with prepuberal characteristics were found to predominate, and the majority had been partially or completely converted into minute sclerotic cords.

In 12 cases the Leydig cells showed a moderate degree of morphological aberration, and in 10 of these cases the cellular hyperplasia was striking (fig. 6*B*). The cells were grouped in large masses or occupied all the interstitial space in those cases in which the tubules were relatively intact. The individual cells and groups of cells were reasonably well defined and did not merge with the cells resembling fibrocytes; the latter were far less numerous than in the first group of cases. However, cytoplasmic secretory droplets, crystalloids and pigment granules were either sparse or absent. Other abnormalities were found in the examination of a group of cells. Often there was a conspicuous variation in cytoplasmic mass, nuclear size and chromatin content. Cells of giant proportions were present, and nuclear gigantism was not an unusual finding. Multinucleation of the cells was commonplace, but this is also true of the normal testis. Plasmosome vacuolation was sometimes encountered.

In the normal testis a small proportion of the Leydig cells seem to be degenerating, and these changes were exaggerated in this group of cases. It was difficult to decide whether this effect was abnormal or merely more conspicuous because large masses of cells in close proximity were being examined. In any event degenerative changes were found in an unusually large number of cells. The cytoplasm of many cells contained large hyaline droplets or was completely hyalinized. Other cells were filled with unusually coarse granules, or the cytoplasm was converted into a fine foam of droplets; these changes were often accompanied by swelling of the cytoplasm and nuclear pyknosis. Anuclear smudges of cytoplasm and cells with ruptured cytoplasmic membranes were frequently encountered.

In these testes the development of the tubules was more advanced than in the group with more marked abnormalities in the interstitial cells. In half the cases the tubules contained mature Sertoli cells, though they were abnormal in respect to spermatogenesis and thickness of the tunica propria, and the sclerotic tubules were of greater diameter than the scarred remnants left by immature tubules. In three cases an occasional tubule showed focal spermatogenic activity with an occasional sperm.

In nine cases the morphologic character of the Leydig cells seemed to be within normal limits, although the number of cells was greatly increased even to the point where the biopsy specimen consisted of little more than a mass of Leydig cells (fig. 7). The cells were disposed in large groups and the cytoplasmic membranes were sharp and well defined. The cytoplasm contained fine and coarse granules and a normal complement of secretory droplets, crystalloids and pigment. In several biopsies, large, highly vacuolated cells were seen to predominate, suggesting pronounced secretory activity (fig. 8). Degenerative phenomena were

relatively difficult to find in these testes, even though large numbers of cells could be examined readily. This fact suggested that the large number of degenerating cells seen in the previous group was abnormal and not merely apparent because more cells could be studied.

In this group of cases the tubules showed the usual changes of the condition. In two cases there was focal spermatogenic activity, and in half the biopsy

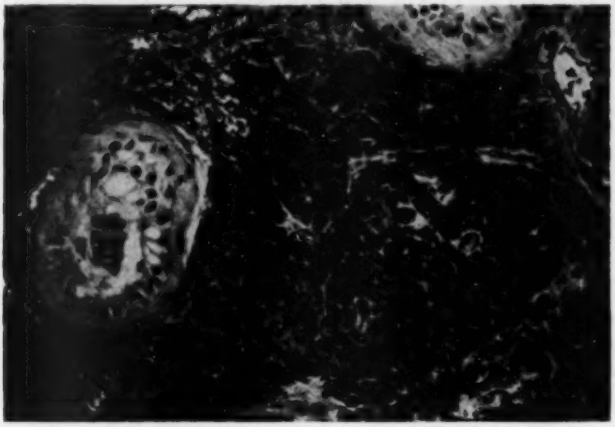

Fig. 7.—Sclerosing tubular degeneration. The hyperplasia of the Leydig cells is impressive. Phosphotungstic acid-hematoxylin stain; × 200.

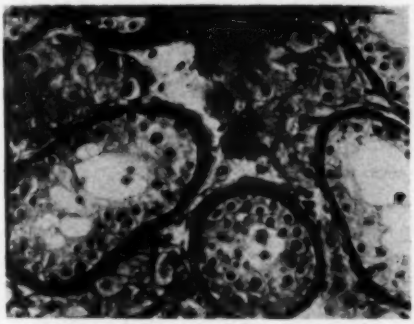

Fig. 8.—Sclerosing tubular degeneration. Note the increased number of morphologically normal Leydig cells. The cytoplasmic membranes are well defined, and the nuclei are uniform, with prominent plasmosomes. The cytoplasm of the cells contains secretory droplets and occasionally crystalloids. The tubules are relatively normal but show a severe degree of hypospermatogenesis. Phloxine-methylene blue stain; × 200.

specimens small sclerotic tubules were present suggesting sclerosis in immature tubules. Case 126, in which the patient was a youth of 17 years, was entered in the group because the Leydig cells seemed qualitatively and quantitatively normal. The tubules were probably within normal limits for the age and showed reasonably active spermatogenesis and no thickening of the tunica propria. However, this

patient had a distinct elevation of follicle-stimulating hormone and gynecomastia. At the moment one cannot predict the subsequent appearance of his testes.

Seven of the patients with gynecomastia had breast tissue removed for cosmetic reasons.[8] In each case the enlarged breast revealed a dense collagen deposit, in the interlobular tissue. There appeared to be no increase in glandular tissue, and the small lobules were widely separated by collagen. Some of the interlobular ducts were dilated. The intralobular ducts showed focal epithelial proliferation with as many as five layers of cells. Sometimes the ducts contained an eosinophilic granular material or a homogeneous basophilic substance like colloid. The intralobular stroma consisted of fine collagen fibrils very lightly sprinkled with plasma cells.

NORMAL TESTES AND HIGH URINARY LEVELS OF FOLLICLE-STIMULATING HORMONE ("CLIMACTERIC")

Six men with decidedly increased excretion of pituitary gonadotropins were believed to have morphologically normal testes. The pertinent data are recorded

TABLE 3.—*"Male Climacteric"*

Case	Age, Yr.	17-Ketosteroids (Urinary), Mg./Day	Follicle-Stimulating Hormone (Urinary), M. U./Day†	Sperm Counts§
131	75	3.9	+192	....
132	55	4.7	+192 +96 −192	....
133	50	10.2	+192	....
134	37	5.7	+104 (D)	24 M.
135	32	9.8	+192	6 M. 15 M.
136	30	7.0	+192	1 M. 60 M.

† For key, see footnote to table 1.
§ For key, see footnote to table 1.

in table 3. Three of these men were in the fourth decade of life, and three were more than 50 years old. The urinary levels of 17-ketosteroids were low, and those of follicle-stimulating hormone were high. The younger men showed oligospermia. The physical development of all the patients was normal, and there was no gynecomastia. The three older men were impotent, and one of them had been disturbed by nervousness and flashes.

In each case the testicular biopsy showed normal tubules with a full complement of gametocytes and sperm, and no abnormalities were detected in the Sertoli cells or in the tunica propria. The variations in the Leydig cells from case to case were considered to be within the normal range, despite unequivocally low 17-ketosteroid excretion in several patients. In case 131, concerning a man of 75 years, the Leydig cells were distributed in small, isolated groups, but many of the cells showed secretory droplets, pigment and crystalloid formation. This peculiar grouping of the cells is not an uncommon pattern in this age group.[1] In case 136 the testis showed a numerical increase in Leydig cells, which were morphologically normal.

COMMENT

Absence of Germ Cells.—No common denominator was found to explain the complete or almost complete absence of spermatogenic cells in the testes of the first group of patients here recorded (19 men). It is known that the germ cells are highly sensitive to their environment and that in the course of many illnesses spermatogenic activity may be depressed. Hypospermatogenesis is a common finding at postmortem examinations after a long or a short illness, especially in the presence of infection and fever.[10] It is possible that various conditions are capable of wiping out the germ cells, and if the spermatogonia are destroyed the testis presumably loses its power to regenerate. An analogy is found in the bone marrow, which a number of agents can suppress irrevocably, even though the agent is removed.

Several authors[11] have recorded similar cases, and recently del Castillo, Trabucco and de la Balze[12] reported five such cases. They believed "that the absence of the germinal epithelium is a result of lack of migration of the primary gonocytes into their definitive place on the urogenital ridge." However, they did not exclude the possibility that the condition is "related to some embryological or post-natal nutritional failure, to some endocrine factor, or to some abnormality of the steroid metabolism." In regard to the failure of migration of the gonocytes, it has not been proved that a "germ track" exists in man, although the evidence for such a mechanism in amphibian and chick embryos is good.[13] If one subscribes to the theory that both Sertoli cells and germ cells arise from the same primitive element within the sex cord, it is possible that the absence of germ cells in the adult gland is the result of abnormal differentiation of the primitive cells.

The tubules of these testes were quite similar to those found in an undescended testis. However, with two exceptions, the patients did not give a history of delayed testicular descent. In each of the exceptions a biopsy of the normally descended testis was made. In an undescended testis, distinctly immature tubules are often scattered throughout the gland, and the Sertoli cells frequently show a type of degeneration in which coarse eosinophilic granules accumulate in the cytoplasm before the final desquamation and disintegration. In these biopsies, all the sustentacular cells seemed to have matured, and signs of degeneration were not found. The undescended testis often contains very large Leydig cells with foamy cytoplasm, and the interstitium may be sclerotic. Similar changes were found in some of the Leydig cells in cases 79 and 95. In the latter the patient did give a history of unilateral maldescent of the opposite testis. Certainly, chosen fields of the testis of a cryptorchid are indistinguishable from the condition under discussion.

Engle[6b] used the term "germinal aplasia" in this connection and stated that the picture is similar to the one found after roentgen radiation to the testes and in

10. Mills, R. G.: J. Exper. Med. **30**:505, 1919. MacLeod, J., and Hotchkiss, R. S.: Endocrinology **28**:780, 1941.

11. Hotchkiss, R. S.: Bull. New York Acad. Med. **18**:600, 1942. Charny, C. W., in Conference on Diagnosis in Sterility, edited by E. T. Engle, Springfield, Ill., Charles C Thomas, Publisher, 1946, p. 43. Simmons, F. A., and Sniffen, R. C.: West. J. Surg. **55**:508, 1947. Engle.[6b]

12. del Castillo, E. B.; Trabucco, A., and de la Balze, F. A.: J. Clin. Endocrinol. **7**:493, 1947.

13. Everett, N. B.: Biol. Rev. **20**:45, 1945.

the cryptorchid testes of late adolescents. His patients gave no history of "cryptorchidism, or radiation, of long febrile disease, or of industrial experience which might be a contributing factor."

Six of the 19 men in this group (cases 89 to 94) had had a good chance of being exposed to unusual amounts of radiant energy. Two patients had had roentgen radiation to the groins in the past for skin diseases. One patient was an x-ray physicist; another was an engineering student who worked with radar during the war. Still another patient was occupied with a cyclotron, and the last patient demonstrated and sold x-ray equipment for a livelihood.

LeRoy [14] and later Liebow, Warren and DeCoursey,[15] reporting on the sequelae of the atomic bomb explosion, illustrated the gradual diminution and final cessation of spermatogenesis, but within a few weeks the "basement membrane" of the tubules showed marked thickening. This change suggested a greater degree of damage than our patients had sustained. Castration cells were found in the pituitary glands of some of the victims.

Five men with evidence of spermatogenesis either in the biopsy or the semen examination have been included in the series. These patients did not differ from the rest in other respects, and indeed several of them showed an increased excretion of pituitary gonadotropins. If it is correct to group the men under one heading, the evidence may indicate that a fertile soil for spermatogenesis is being maintained in the testis, that some agent has completely or almost completely destroyed the sensitive germ cells and that the lesion is not the result of defective development of the testis.

The authors feel that the disease primarily affects the germ cells. The Leydig cells were considered to be normal in 15 patients and numerically increased in four. No abnormality was detected in the cells morphologically, with the exception of the two patients mentioned above in whom a small number of large, foamy interstitial cells were found. There was an increase in cells resembling fibrocytes in the interstitial tissue of seven patients. Four of these men had been exposed to radiant energy and 2 more patients with lesser degrees of fibrosis gave a history of maldescent of the contralateral testis (cases 95 and 96).

Sclerosing Tubular Degeneration.—In the original report on sclerosing tubular degeneration Klinefelter, Reifenstein and Albright [5] emphasized certain points in the condition. All the men were said to have passed through a normal puberty from a clinical standpoint, though the younger subjects appeared immature at the time of the study. No history of maldescent of the testes or of orchitis could be elicited, but each man said that his testes had always been small. Gynecomastia had been noticed by the patients one to six years after the beginning of puberty, and on physical examination there was areolar enlargment with very little increase in pigmentation; no secretion could be obtained from the breast. The authors believed that the histologic appearance of the Leydig cells was normal and that the numerical increase of these cells was due to tubular sclerosis and a consequent

14. LeRoy, G. V.: Medical Sequelae of Atomic Bomb Explosion, J. A. M. A. **134**:1143 (Aug. 2) 1947.

15. Liebow, A. A.; Warren, S., and DeCoursey, E.: Am. J. Path. **25**:853, 1950.

decrease in the volume of the glands. As their patients did not show striking signs of eunuchoidism, it was felt that the function of the Leydig cells was almost adequate.

In 1945 Heller and Nelson [9a] reported their findings in 20 men with similar endocrine abnormalities. They enlarged the scope of the original syndrome defined by Klinefelter, Reifenstein and Albright, and included those patients who had atrophic testes with hyalinization of the tubules and clumping of the Leydig cells, azoospermia and elevation of the urinary gonadotropins. On the basis of variable features their patients were further classified as eunuchoidal, moderately eunuchoidal or normal. The variable features included the bone age, the occurrence of gynecomastia, the development of the external genitalia, the hair pattern, the pitch of the voice, the muscular development, the quality of the Leydig cells, the 17-ketosteroid excretion and the excretion of estrogens. This study demonstrated that gynecomastia does not always develop in the presence of severe tubular sclerosis and high levels of urinary gonadotropins and that patients with these abnormalities may be distinctly eunuchoid.

In a companion paper Nelson and Heller [9a] reported the histopathological abnormalities observed in the testes and the breasts of their patients. They believed that in the testis the primary defect lay in the seminiferous tubules, in which there was a progressive depostion of collagen in the lamina propria and the basement membrane, resulting in retardation of spermatogenesis and finally in complete tubular sclerosis. An increase in the number of Leydig cells and a tendency to form large clumps were evident in most of the biopsies; the numerical increase in cells was attributed to loss of testicular volume. Great stress was placed on the appearance of the Leydig cells, and especially on the number of refractile granules contained in the cytoplasm, which they felt were closely related to the functional activity of the cells. In most instances the Leydig cells appeared "to be undergoing functional failure" and the fibroblasts and "fat cells" of the interstitial tissue seemed to be increasing at the expense of the Leydig cells. The authors felt that it was "possible to correlate the condition of the Leydig cells with the clinical condition of the patients and, in a general fashion, with the output of 17-ketosteroids." The functional performance of the Leydig cells was judged primarily from an estimation of the degree of cytoplasmic granulation, with birefringency, the Schultz reaction for cholesterol and sudanophilia as supplementary tests.

In the present study it was evident that the patients with "sclerosing tubular degeneration" had one point in common—an increased excretion of pituitary gonadotropins. If one eliminated case 126 from the group, in which an early stage of the condition may have been observed, these men had another point in common—abnormal testes. The other features of the condition, such as gynecomastia, eunuchoidism and 17-ketosteroid excretion, were variable. There were indications that the disease was present at puberty. Possibly the defect may be carried from generation to generation, as the patients in cases 105 and 106 were brothers and the patient in case 104 was their uncle. The family has been reported by Reifenstein.[16]

The sequence of events that leads to progressive tubular sclerosis during or shortly after puberty is not known. As far as one can tell, the pituitary is not

16. Reifenstein, E. C., Jr.: Proc. Am. Federation Clin. Research **3**:86, 1947.

at fault, and there is no evidence that the condition is the result of a nutritional deficiency, of exogenous or endogenous toxins or of remote disease. The fact that the syndrome has been found in several members of a family should provide a lead for future reference. There is no suggestion in the publications on the condition that an inflammatory reaction is present in the testes. It is possible that the events occur in the following sequence. First the Sertoli cells atrophy, and thereupon spermatogenesis ceases. As activity diminishes within the tubule, collagen is deposited within the tunica propria until the tubule finally becomes a sclerotic cord. It has been demonstrated many times that the germ cells can disappear from the tubule without apparent change in Sertoli cells, basement membrane or tunica propria, and furthermore there need not be a derangement in hormonal relationship. Atrophy of the Sertoli cells is always accompanied by thickening of the tunica propria. Nelson and Heller[6a] expressed the belief that the atrophic changes in the germinal epithelium are secondary to sclerosis of the basement membrane and the lamina propria. It is equally possible that the tunica thickens because the tubule is functionless after the Sertoli cells have failed. The sclerosis of a large mass of tubular tissue might also explain the thickening of arterial walls occasionally observed in the testes of these patients. Atrophy occurring in other organs for any reason regularly leads to thickening of the arterial walls. The tubular sclerosis in this condition appears to differ from the focal tubular atrophy that occurs in apparently healthy men and in senility. In the latter instances there is an increase in fibrocytes, collagen and elastic fibrils in and around the outer lamellas of the tunica propria, while the tubules under discussion show collagen deposition in the inner tunica without an increase in elastic fibrils. Mott[17] and Hemphill, Reiss and Taylor[18] described the picture in the testes of young men suffering from schizophrenia, in which the tubular change appears to be somewhat similar to that found in sclerosing tubular degeneration. It is interesting that these authors noted that there was no increase in the Leydig cells despite severe tubular sclerosis—and the latter group thought there were no morphological changes in the Leydig cells. Such statements strengthen our opinion that there is an absolute increase in interstitial cells in the present condition. There was no obvious relation between the degree of damage to the tubules, the level of pituitary gonadotropin excretion and the physical development of the patient. Gynecomastia was an irregular finding, whether or not the patient was eunuchoid. There did seem to be some relationship between the degree of Leydig cell abnormality, the level of the 17-ketosteroids and the physical development of the patient, as illustrated in table 4. The point is not clearcut, but as the condition of the Leydig cells improved, the average excretion of 17-ketosteroids increased and a greater proportion of patients in each group showed less marked eunuchoidism.

The male climacteric is a nebulous condition that seems to be rather uncommon.[19] It is unusual for elderly men to have elevated urinary gonadotropins, while it is the rule in postmenopausal women. Nevertheless, some men do suffer from symptoms similar to those experienced by women during the menopause.

17. Mott, F. W.: Brit. M. J. **2**:737, 1919.

18. Hemphill, R. E.; Reiss, M., and Taylor, A. L.: J. Ment. Sc. **90**:681, 1944.

19. Werner, A. A.: Male Climacteric, J. A. M. A. **112**:1441 (April 15) 1939. McCullagh, E. P.: Cleveland Clin. Quart. **13**:166, 1946. Werner, S. C.: Am. J. Med. **3**:52, 1947.

Heller and Myers [20] and Nelson [21] studied a group of these patients and found that they fell into two major categories. One group showed no hormone imbalance and no response to hormone therapy. They believed these men to be psychoneurotic. The other group showed elevated excretions of urinary gonadotropins and responded favorably to intramuscular testosterone propionate. Eight of the 23 men in their series with high values for follicle-stimulating hormone underwent testicular biopsies. Three of them were shown to have sclerosing tubular degeneration (Klinefelter's syndrome), and in the remaining five there were "reduction in size and in activity of the seminiferous tubules and reduction in size and number

TABLE 4.—*Correlation of Observations in Cases of Sclerosing Tubular Degeneration*

Case	Condition of Leydig Cells	17-Ketosteroids (Urinary), Mg./Day	Habitus of Patient
98	Poor	7.2	Little eunuchoidism
99	Poor	10.3	Little eunuchoidism
101	Poor	3.4	Little eunuchoidism
103	Poor	5.1	Little eunuchoidism
108	Poor	10.1	Little eunuchoidism
119	Poor	4.6	Marked eunuchoidism
121	Poor	6.6	Marked eunuchoidism
122	Poor	4.0	Marked eunuchoidism
124	Poor	3.5	Marked eunuchoidism
125	Many fibrocytes—no Leydig cells	0.9	Age 14—gynecomastia
102	Fair	7.2	Little eunuchoidism
104	Fair	8.8	Little eunuchoidism
105	Fair	14.7	Little eunuchoidism
106	Fair	13.3	Little eunuchoidism
109	Fair	9.0	Little eunuchoidism
110	Fair	9.6	Little eunuchoidism
113	Fair	4.9	Little eunuchoidism
116	Fair	N. D.	Little eunuchoidism
117	Fair	4.7	Marked eunuchoidism
118	Fair	1.8?	Marked eunuchoidism
123	Fair	7.0	Marked eunuchoidism
126	Fair	7.0	Age 17—gynecomastia
97	Good	13.1	Little eunuchoidism
100	Good	11.7	Little eunuchoidism
107	Good	16.3	Little eunuchoidism
111	Good	8.6	Little eunuchoidism
112	Good	N. D.	Little eunuchoidism
114	Good	12.0	Little eunuchoidism
115	Good	9.1	Little eunuchoidism
120	Good	6.3	Marked eunuchoidism

of Leydig cells. The latter were abnormal in granulation and lipid content." The youngest patient who fell into the category of "male climacteric" was 25 years old. We have not been able to demonstrate abnormalities in the testes of men with similar symptoms and elevated urinary gonadotropins.

In the three conditions under discussion it was felt that a defective pituitary was not responsible for the abnormalities in these men and that the fault originated

20. Heller, C. G., and Myers, G. B.: J. Clin. Investigation **21**:622, 1942; Male Climacteric, Its Symptomatology, Diagnosis and Treatment: Use of Urinary Gonadotropins, Therapeutic Tests with Testosterone Propionate and Testicular Biopsies in Delineating Male Climacteric from Psychoneurosis and Psychogenic Impotence, J. A. M. A. **126**:472 (Oct. 21) 1944.

21. Nelson, W. O.: J. Omaha Mid-West Clin. Soc. **8**:1, 1947; M. Clin. North America **32**:97, 1948.

in the gonads. The clinical and laboratory details and the various hypotheses that attempt to explain the hormone imbalance in these patients are reported in another article.[3]

SUMMARY

Observations were made on the testicular abnormalities of 55 men with hormonal imbalance manifested by increased excretion of urinary gonadotropins and, in certain ones, by low output of urinary 17-ketosteroids and signs of eunuchoidism.

In the first group of patients spermatogenic activity had usually failed completely, but since the hormone-producing elements of the testis, the Sertoli cells and the Leydig cells, were intact, evidence of endocrine derangement was minimal, although sometimes definite with regard to the excretion of urinary gonadotropins. It is felt that the sensitive germ cells were destroyed by some agent operating in the past and that the condition is not the result of maldevelopment of the testis.

The second group of patients showed progressive sclerosis of the seminiferous tubules associated with hyperplasia of the Leydig cells, the latter often exhibiting morphological abnormalities. These changes were accompanied with an increased excretion of urinary gonadotropins and often a diminished output of 17-ketosteroids. There were definite indications that the disease began at puberty. It is suggested that the primary abnormality lies in the Sertoli cells or the Leydig cells and that the resulting hormonal imbalance leads to a disturbance in pituitary function and frequently to signs of eunuchoidism and gynecomastia.

In a third group of patients the findings were consistent with the so-called "male climacteric." In this syndrome the urinary gonadotropins were significantly elevated, but the testes seemed to be normal as judged from routine histological preparations.

HISTOPATHOLOGY OF EXPERIMENTAL HISTOPLASMOSIS

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OPPORTUNITY to study the lesions produced by Histoplasma capsulatum in experimental animals was recently provided in studies on this organism. In the experiments the susceptibility of various kinds of mice inoculated with several strains of Histoplasma by various routes was investigated.[1]

This report is based on gross and microscopic examinations of 290 male and 70 female dba line 1 mice, 23 male and 22 female BHC57B10 mice and 43 male and 43 female BHC57B6 mice. Control animals for each route of inoculation were available.

Complete autopsies were performed on all the mice. The tissues were fixed in Zenker's solution, and sections were cut at 6 microns. They were stained with hematoxylin and eosin and Goodpasture's bacterial stain.

In the dba line 1 male mice the injections were made as follows: intracerebrally in 226, intravenously in 19, intraperitoneally in 20, with the organisms suspended in saline solution, and intraperitoneally in 25, with the organisms suspended in mucin. In the dba line 1 female mice the injections were made intracerebrally in 45 and intraperitoneally in 9, with the organisms suspended in mucin.

Of the BHC57B10 mice, 11 males and 10 females received the organisms by the intracerebral route and 12 males and 12 females by the intraperitoneal route. The organisms were suspended in mucin.

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1. Howell, A., Jr.; Kipkie, G. F., and Bruyere, P. T.: Studies on Experimental Histoplasmosis: I. A Report on Intracerebral Inoculations of Male dba Line 1 Mice, Extracts from Pub. Health Rep. **65**:722-735, 1950. Howell, A., Jr., and Kipkie, G. F.: Studies on Experimental Histoplasmosis: II. A Comparison of the Susceptibility by Intracerebral Inoculation of Six Strains of Mice with Male dba Line 1 Mice, Am. J. Trop. Med., to be published; III. Experimental Histoplasmosis: The Susceptibility of Male dba Line 1 Mice by Various Routes of Injection, Proc. Soc. Exper. Biol. & Med. **75**:121-123, 1950; IV. Studies on Experimental Histoplasmosis: A Comparison of the Virulence of Five Strains of Histoplasma Capsulatum by Intracerebral Inoculation of Male dba Line 1 Mice, J. Lab. & Clin. Med. **36**:547-554, 1950.

Thirty-one male and 31 female BHC57B6 mice received the organisms intracerebrally, and 12 males and 12 females intraperitoneally, with the organisms suspended in mucin.

GROSS LESIONS

The lesions noted in the gross examinations depended on the route of inoculation. Within the individual groups the changes observed were similar, regardless of the strain of mice, the sex of the animals, whether the animals died or were killed and regardless of the strain of H. capsulatum employed. There were relatively insignificant variations in the severity of the lesions produced.

Intracerebral Inoculation.—The animals receiving intracerebral injections showed no gross lesions whether they died or were killed. Occasionally a spleen appeared to be slightly larger than usual, but this finding was recorded also in the control animals and therefore was felt to be of no consequence.

Intravenous Inoculation.—Those mice which received intravenous injections had gross lesions of the lungs, the liver and the spleen. The lesions of the lungs appeared as pale grayish white areas of discoloration which measured up to 2 mm. in diameter. Similar areas of discoloration were present also in the liver and the spleen. The colors of these two organs had changed from the usual reddish brown or reddish purple to a pale grayish brown. The livers and spleens were enlarged, the spleens showing the greater enlargement, sometimes measuring 10 times normal size. The largest spleens encountered in all the experiments were in the animals in which a Histoplasma suspension had been injected by the intravenous route.

Intraperitoneal Inoculation with Organisms Suspended in Saline Solution.—The mice into which the organisms suspended in saline solution were injected had minimal adhesions in the peritoneal cavity. The livers and the spleens were adherent to the abdominal wall and to the surrounding viscera. Both organs were enlarged, the enlargement reaching its maximum degree in the spleen, which was usually five to 10 times normal size. The color of both organs was a dusky pale grayish brown. Only rarely were any flecks of creamy yellow exudate present on peritoneal surfaces. Slightly thickening was present in the groins at the sites of intraperitoneal injection, but no gross lesions were noted in the lungs or the brain in any of this group.

MICROSCOPIC LESIONS

Intracerebral Inoculation.—The lesions to be described, in the main, occurred in animals dying spontaneously and those killed on the thirtieth day after injection. In the brain (fig. 1 *A*) the lesions consisted of a mononuclear cell infiltration beginning in the meninges and extending into the deeper portions of the brain along perivascular spaces. The exudate varied from a mild lymphocytic infiltration to an extensive granulomatous inflammation in which the macrophages contained phagocytosed Histoplasma. Rarely the granulomas had an epithelioid appearance and giant cells were encountered. Occasionally a few polymorphonuclear granulocytes were present with the mononuclear cells. Occasionally, masses of organisms were present without any cellular reaction. These were found either in the subarachnoid space or deep in the cerebral tissue.

The lesions of the liver consisted of varying degrees of reticuloendothelial hyperplasia with focal necrosis of liver cells. These focal involvements were concentrated

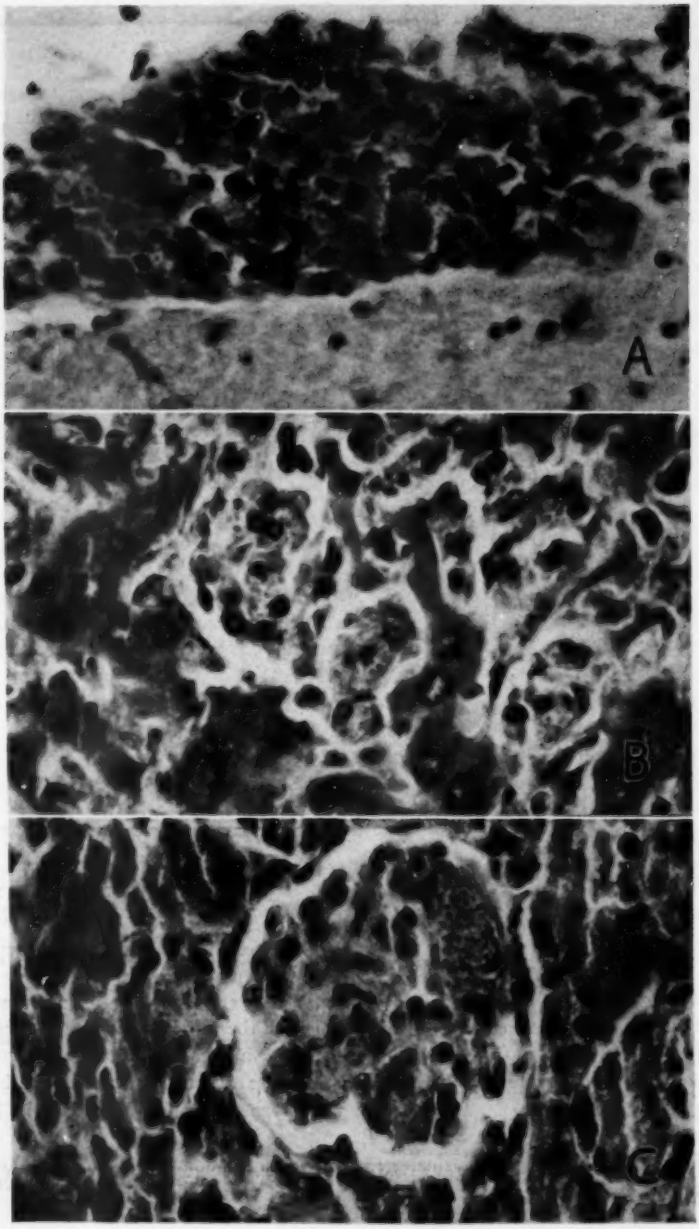

Fig. 1.—*A*, pure granulomatous inflammation in meninges. Mouse inoculated intracerebrally with Histoplasma capsulatum. Numerous organisms are present. Hematoxylin and eosin stain; × 888.

B, hyperplasia of reticuloendothelial cells of liver. Kupffer cells contain organisms. The mouse was inoculated intravenously with Histoplasma. Hematoxylin and eosin stain; × 888.

C, kidney with Histoplasma occupying a portion of a glomerular tuft. No cellular reaction is present. The mouse was inoculated intravenously with Histoplasma. Hematoxylin and eosin stain; × 888.

chiefly about the central veins. Rarely a central vein contained thrombotic material. Mild to moderate mononuclear cell infiltrations were seen in the areas of necrotic liver. Histoplasma was present in the Kupffer cells and the liver cells themselves.

The splenic lesions appeared as a proliferation of reticuloendothelial cells. In some animals this was diffuse and extensive, while in others it was patchy. Rarely areas of necrosis were present. Organisms were found in the hyperplastic reticuloendothelial cells.

Other lesions were rarely found. Occasionally Histoplasma would be seen in the sinusoids of the lymphoid tissue of the gastrointestinal tract, in the spinal canal, apparently extending from the brain, and in the heart. The lesions of the heart were myocardial necrosis, fibrosis and calcification with a few infiltrating mononuclear cells. Both ventricles were involved in about equal frequency.

Intravenous Inoculation.—Only dba line 1 male mice received injections of H. capsulatum by the intravenous route. Of these 19 mice, three died. All three had lesions in the liver, spleen, adrenal, lungs and heart; two had lesions in the pancreas, kidney and testis, and one had lesions in the brain. Of the 16 killed on the thirtieth day after injection, one had a questionable lesion in the brain, six had lesions in the liver, five in the spleen, four in the heart, three in the lungs, one in the pancreas and one in the lymph nodes.

The intra-abdominal lesions consisted of marked diffuse reticuloendothelial hyperplasia of liver (fig. 1 *B*) and spleen. Phagocytosed organisms were present in these cells. In the lung, the pancreas and the testis the lesions consisted of necrotic granulomas with varying numbers of polymorphonuclear granulocytes intermingled. The organisms were present both free and inside macrophages. The lesions of the brain and heart were similar to those in the intracerebral injection group except that the lesions of the brain were of a very mild character. In the kidney the organisms were present in large numbers in interlobular arteries and filling portions of glomerular tufts (fig. 1 *C*). Occasionally they were seen in the tubules also. The cellular reaction excited by the fungus in these areas was minimal or absent. The difference between the lesions in animals dying spontaneously and those killed was one of quantity, not quality.

Intraperitoneal Inoculation with Organisms Suspended in Saline Solution.—This type of injection was also restricted to dba line 1 mice. Most animals dying spontaneously had lesions in the liver and the spleen, the site of inoculation and the pancreas. No animal had a lesion in the brain. In the killed group, again the majority had lesions in the liver, the spleen, the site of inoculation and the pancreas; but lesions were also seen in the lungs, the heart and the intestine in a few animals. However, no lesion was present in the brain of any animal in the killed group.

The most prominent feature was the extensive granulomatous process present on the surfaces of the viscera. These granulomatous areas usually had a necrotic center in which organisms were frequently visible. Surrounding the necrotic areas the macrophages occasionally took on an epithelioid character (fig. 2 *A*). Rarely a giant cell was encountered. The macrophages contained numerous organisms. Scant collections of lymphocytes and plasma cells were found at the periphery of the macrophage accumulations. In the liver and the spleen, the surface granulomas merged with the underlying parenchymal lesions. Foci of necrotic liver, usually around 50 microns in diameter, but occasionally larger, were scattered throughout, and the

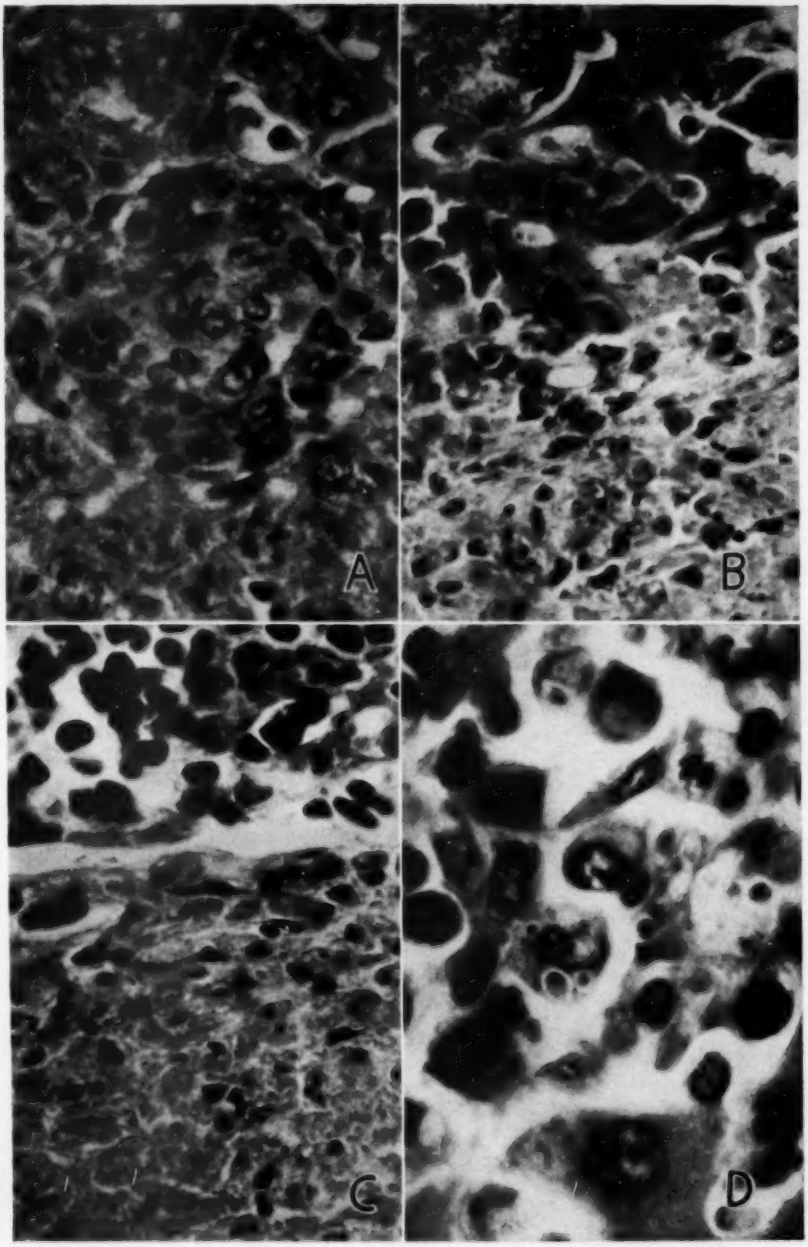

Fig. 2.—*A*, granuloma in liver showing macrophages of an epithelioid character. The mouse was inoculated intraperitoneally with Histoplasma organisms suspended in saline solution. Hematoxylin and eosin stain; × 888.

B, reticuloendothelial hyperplasia of liver with organisms present. Granuloma was present on the surface of the organ. The mouse was inoculated intraperitoneally with Histoplasma organisms suspended in mucin. Hematoxylin and eosin stain; × 888.

C, necrotic granuloma at the site of an intraperitoneal inoculation of a mouse with Histoplasma organisms suspended in mucin. The organisms are present. Hematoxylin and eosin stain; × 888.

D, liver showing detail of Histoplasma capsulatum inside Kupffer cells. The mouse was inoculated intraperitoneally with organisms suspended in mucin. Goldman stain; × 2175.

reticuloendothelial cells were notably hyperplastic. Histoplasma organisms were seen in the reticuloendothelial cells and in apparently viable liver cells. Necrosis was uncommon in the spleen, the lesion being seen as a prominent reticuloendothelial hyperplasia with presence of the organisms. These basic pathologic-anatomic changes were observed in the other viscera listed.

Intraperitoneal Inoculation with Organisms Suspended in Mucin.—Seventy-nine animals received injections by the intraperitoneal route. The lesions in the males were slightly more severe than those in the females. The strain of mice made no difference in the lesions which appeared in those dying spontaneously and in those killed on the thirtieth day after injection. The difference between this group and the group which received the injections by the same route with the organisms suspended in saline solution was one of degree. Here there was much more exudate on surfaces, the granulomatous lesions of the lungs were more extensive and the reticuloendothelial hyperplasia of the liver (fig. 2 *B*) and the spleen was more outstanding with a minimal necrotic component. Similar granulomatous lesions were seen at the sites (fig. 2 *C*) of inoculation. In one of the 79 animals a lesion developed in the brain. This was a granulomatous reaction in which the organisms were found.

Material from an occasional animal in this group was stained with Goldman's [2] stain. This was originally designed to aid in the identification of Leishmania donovani and Trypanosoma cruzi in tissue sections. The stain also lends itself to the recognition of H. capsulatum in tissues. It is an iron alum-picric acid-hematoxylin stain. With it each of the parasitic cells is surrounded by a very definite clear halo (fig. 2 *D*). The central chromatin may be a solid mass, may be subdivided into two or more deeply black-staining masses which are separated by clear areas or may be finely dispersed throughout the cell. Many of the organisms appeared spherical, while others were oval. Budding organisms were not seen in any of the tissues.

No gross or microscopic lesions were found in any of the control animals.

COMMENT

From a consideration of these tissues it would seem that H. capsulatum is to be cataloged as one of the agents causing a chronic granulomatous reaction in the tissues of the host. This is in essential agreement with the observations of other writers.[3]

The organs most commonly involved are the liver and the spleen, regardless of the route of inoculation. The invading organisms do not always stimulate a granulomatous reaction, as frequently they are seen lying free in tissues without any inflammatory cells in the immediate neighborhood.

When correlated with the various routes of injection, the distribution of the lesions would seem to bear a direct relation to the number of parasites entering

2. Goldman, M.: (*a*) An Iron Alum-Picric Acid-Hematoxylin Stain for Parasites in Tissues, J. Parasitol. (supp. 6) **35**:90, 1949; (*b*) Stain Technol., to be published.

3. Parsons, R. J.: Experimental Histoplasmosis in Mice, Arch. Path. **34**:229 (July) 1942. Tager, M., and Liebow, A. A.: Observations on Histoplasmosis: Induced Infection in the Mouse, Yale J. Biol. & Med. **14**:469, 1942. Emmons, C. W., and Ashburn, L. L.: Histoplasmosis in Wild Rats: Occurrence and Histopathology, Pub. Health Rep. **63**:1416, 1948.

the general circulation. By the intracerebral route the brain, the liver and the spleen were involved in most cases, although the organisms were frequently hard to find. Other organs were involved less frequently. The most widespread lesions were noted in the animals in which the fungus was injected intravenously. Granulomas were scattered throughout the entire body, although the brain did remain free of infection. Many of the granulomas showed centers of necrosis surrounded by areas of macrophages, some of which were epithelioid in type, rare giant cells and a peripheral scattering of lymphocytes.

The lesions in the intraperitoneally inoculated groups varied only in degree. The reason there were more necroses in the liver in the group in which the organisms were suspended in saline solution may be that these animals lived longer than the group in which the organisms were suspended in mucin, so that necroses had time to develop. It is not possible to state that mucin had a protective action on the fungous cells in inhibiting their destruction. It did not interfere with phagocytosis, as this was a conspicuous feature of the lesions in this group of animals. However, these organisms may have survived and grown for a longer time after phagocytosis.

SUMMARY

Histological studies were performed on a total of 491 dba line 1, BHC57B10 and BHC57B6 male and female mice following intracerebral, intravenous and intraperitoneal injection of various strains of Histoplasma capsulatum. The intraperitoneal injection group was divided into one group in which the organisms were suspended in saline solution and a second in which they were suspended in mucin.

The lesions produced are regularly and typically granulomatous. The Goldman iron alum-picric acid-hematoxylin stain is especially useful for demonstrating these parasites in the tissues.

MINERALS OF NORMAL AND ATHEROSCLEROTIC AORTAS

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IN A PREVIOUS publication Buck and Rossiter [1] described the lipid composition of the human aorta in relation to aging and to the degree of atherosclerosis. As a result of aging, apart from atherosclerosis, there was an increase in the concentrations of total lipid, total phospholipid and total cholesterol. The increase in the concentration of phospholipid was entirely the result of an increase in the concentration of sphingomyelin. As a result of increasing severity of atherosclerosis, apart from aging, there was also an increase in the concentrations of total lipid, total phospholipid and total cholesterol. In this instance the increase in the concentration of phospholipid was the result of an increase in the concentrations of both sphingomyelin and lecithin but not of cephalin. Evidence was presented to support the view that the lipids deposited in the aorta as the result of aging alone are deposited in the media, while those associated with the degree of atherosclerosis are deposited in the intima. The present publication deals with the concentrations of nonlipid phosphorus, calcium, magnesium, fat-free dry residue and water in the same autopsy material.

Gazert [2] appears to have been the first to carry out a quantitative chemical study of the mineral content of the human aorta. Since that time numerous reports have appeared. The similarity between calcification and ossification was noted [3]; the media was found to be the chief site of calcification [4]; the process of aging was described as having an important influence on the mineral content of the

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This investigation was supported by a grant from the National Research Council of Canada.

1. Buck, R. C., and Rossiter, R. J.: Lipids of Normal and Atherosclerotic Aortas: A Chemical Study, Arch. Path. **51**:224 (Feb.) 1951.

2. Gazert: Deutsches Arch. f. klin. Med. **62**:390, 1899.

3. (*a*) Baldauf, L. K.: J. M. Research **15**:355, 1906. (*b*) Wells, H. G.: ibid. **14**:491, 1906; (*c*) Calcification and Ossification, Arch. Int. Med. **7**:721 (June) 1911; (*d*) in Cowdry, E. V.: Arteriosclerosis: A Survey of the Problem, New York, The Macmillan Company, 1933, chap. 11. (*e*) Schoenheimer, R.: Ztschr. f. physiol. Chem. **177**:143, 1928. (*f*) Frondel, C., and Prien, E. L.: Science **103**:326, 1946.

4. (*a*) Mönckeberg, J. G.: Virchows Arch. f. path. Anat. **171**:141, 1903. (*b*) Ravault, P.: Bull. d'histol. appliq. à la physiol. **5**:40, 1928. (*c*) Policard, A.; Morel, A., and Ravault, P. P.: Compt. rend. Acad. d. sc. **194**:201, 1932. (*d*) Weinhouse, S., and Hirsch, E. F.: Chemistry of Atherosclerosis: Lipid and Calcium Content of Intima and of Media of Aorta With and Without Atherosclerosis, Arch. Path. **29**:31 (Jan.) 1940. (*e*) Blumenthal, H. T.; Lansing, A. I., and Wheeler, P. A.: Am. J. Path. **20**:665, 1944. (*f*) Lehnherr, E.: New England J. Med. **208**: 1307, 1933. (*g*) Klotz, O.: J. Exper. Med. **7**:633, 1905.

aorta,[5] and the relation of calcification of the aorta to atherosclerosis was variously described.[6]

In the present investigation an attempt has been made to express quantitatively the relation of the concentrations of nonlipid phosphorus, calcium and magnesium to the two factors that have been found to be particularly important in determining other chemical changes in the aorta: (1) aging and (2) the pathological process of atherosclerosis.

METHODS

The concentrations of minerals were determined in a total of 55 pieces of aorta taken at autopsy from 30 subjects whose ages ranged from birth to 85 years. The adventitia was stripped from each piece of aorta, and the remaining intima and media rapidly weighed (1 to 3 Gm.). If the aorta appeared normal on gross examination, one piece of tissue only was taken. If the aorta appeared atherosclerotic, one piece of tissue was taken from a region that appeared normal on gross examination and one or two additional pieces were selected to represent various degrees of atherosclerosis seen in the intima.

From each piece of aorta the lipids were extracted as described previously.[1] The remaining tissue was dried for several days at 37 C. and is referred to as the fat-free dry residue. This residue was weighed, after which it was heated for 24 hours, first with nitric acid and then with perchloric acid according to the method of Buell.[7] The volume of this clear digest was made up to 100 cc., and suitable aliquots were taken for the determination of the concentrations of nonlipid phosphorus, calcium and magnesium. All estimations were made in duplicate.

Analytical Methods.—Nonlipid phosphorus was measured by a modification of the method of Fiske and Subbarow[8] described by King.[9]

Calcium was determined by a method incorporating suggestions of DeLuca[10] and Polley.[11] The calcium was precipitated at p_H 3 to 4 as oxalate (2,4-dinitrophenol indicator) by the addition of 0.2 cc. of saturated ammonium oxalate. The tube was centrifuged for 15 minutes at 3,000 revolutions per minute, and the supernatant solution was decanted off and saved for the determination of magnesium. The precipitate was washed with a mixture of water, alcohol and ether (2:2:1), dissolved in twice-normal sulfuric acid, and oxidized at 70 C. by an excess of 0.002 normal ceric sulfate. Color was developed with 5 per cent potassium iodide. Standard solutions containing 50, 100 and 150 μg. of calcium were carried through the procedure. The intensity of the color was read in a Coleman Universal Spectrophotometer at 400 mμ. against a blank containing no ceric sulfate.

Magnesium was measured as described by Heagy.[12] The method consists of the colorimetric determination of a specific red color formed in the reaction between magnesium hydroxide and titan yellow.

The water content of the tissue was calculated as follows: Water = fresh weight of tissue — (total lipid + fat-free dry residue).

Assessment of Degree of Atherosclerosis.—The details of the assessment of the degree of atherosclerosis were given in the previous publication.[1] Each piece of aorta was classified into one of four grades: grade 0 (no evidence of atherosclerosis); grade 1 (discrete plaques not over 2 mm. in diameter); grade 2 (discrete or confluent plaques not over 2 mm. in diameter but with no evidence of necrosis or ulceration); grade 3 (as in grade 2 but with necrosis and/or

5. (*a*) Bürger, M.: Ztschr. f. d. ges. Neurol u. Psychiat. **167**:273, 1939. (*b*) Kirk, E., and Kvorning, S. A.: Am. Heart J. **38**:476, 1949. (*c*) Faber, M., and Lund, F.: The Human Aorta: Influence of Obesity on the Development of Arteriosclerosis in the Human Aorta, Arch. Path. **48**:351 (Oct.) 1949. Gazert.[2] Weinhouse and Hirsch.[4d]

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9. King, E. J.: Biochem. J. **26**:292, 1932.

10. DeLuca, H. A.: Canad. J. Research Sect. B **25**:449, 1947.

11. Polley, J. R.: Canad. J. Research, Sect. E **26**:188, 1948.

12. Heagy, F. C.: Canad. J. Research, Sect. E **26**:295, 1948.

ulceration). A quantitative evaluation of the degree of atherosclerosis was obtained by measuring the thickness of the intima from the internal elastic lamina to the lumen. This was done in stained histological sections with the aid of a micrometer eyepiece.

RESULTS

Concentrations of Minerals.—Table 1 shows the mean concentrations of nonlipid phosphorus, calcium, magnesium, fat-free dry residue and water for each of the grades as determined by gross examination. The standard error is shown below each mean. The table also gives the mean thickness of the intima and the mean age of the subjects for each grade. The coefficient of correlation (r) between the degree of atherosclerosis (as determined by the gross grading) and the concentration of each substance was calculated by the method of group correlation. The significance of each value of r can be judged from its standard error ($S.E.r$) and the value of P given at the foot of each column.

TABLE 1.—*All Aortas—Mean Concentrations of Nonlipid Phosphorus, Calcium, Magnesium, Fat-Free Dry Residue and Water in the Pieces of Aorta Classified According to the Degree of Atherosclerosis*

Gross Grade	N*	Mean Age, Yr.	Mean Thickness of Intima, Microns	Mean Concentration†				
				Nonlipid Phosphorus	Calcium	Magnesium	Fat-Free Dry Residue	Water
0	17	27	126 ± 17	0.085 ±0.017	0.120 ±0.035	0.011 ±0.003	22.15 ± 0.87	76.46 ± 0.88
1	11	54	352 ± 62	0.116 ±0.024	0.212 ±0.047	0.015 ±0.002	20.39 ± 0.63	77.75 ± 0.60
2	14	64	1309 ±169	0.309 ±0.122	0.801 ±0.292	0.029 ±0.007	19.85 ± 0.75	74.72 ± 0.95
3	13	67	2068 ±378	1.238 ±0.323	3.003 ±0.912	0.046 ±0.013	22.75 ± 2.15	68.99 ± 2.83
r.	..	..	0.76	0.66	0.62	0.46	0.20	—0.46
S. E. r.	..	..	±0.06	±0.08	±0.08	±0.11	± 0.13	± 0.11
P	..	..	< 0.01	< 0.01	< 0.01	< 0.01	> 0.1	< 0.01

* N is the number of pieces of aorta.
† The concentrations are expressed as mg. per 100 mg. wet tissue ± S. E. mean.

The concentrations of nonlipid phosphorus, calcium and magnesium increased with each successive grade. The concentration of water decreased slightly, while that of the fat-free dry residue changed very little. However, the problem was to determine the relative importance of the two factors (1) age, which was progressively greater in each grade, and (2) the degree of atherosclerosis, indicated on the table by the intimal thickness, which also increased progressively.

Relation to Age.—In order to eliminate the effect of atherosclerosis, the concentrations of minerals, fat-free dry residue and water were studied separately in those pieces of aorta that showed no evidence of atherosclerosis on gross examination (grade 0).

For the minerals it was found (table 2) that, although the concentration in terms of fresh weight of tissue was significantly correlated with age, the correlation of the logarithm of the mineral concentration with age was of greater significance. In order to avoid negative values for the logarithms, the concentration of each mineral was multiplied by 1,000 and the logarithm of this number taken. The relation of the logarithm of the concentration of each mineral to age is shown in chart 1. The solid line represents the calculated regression line and the broken

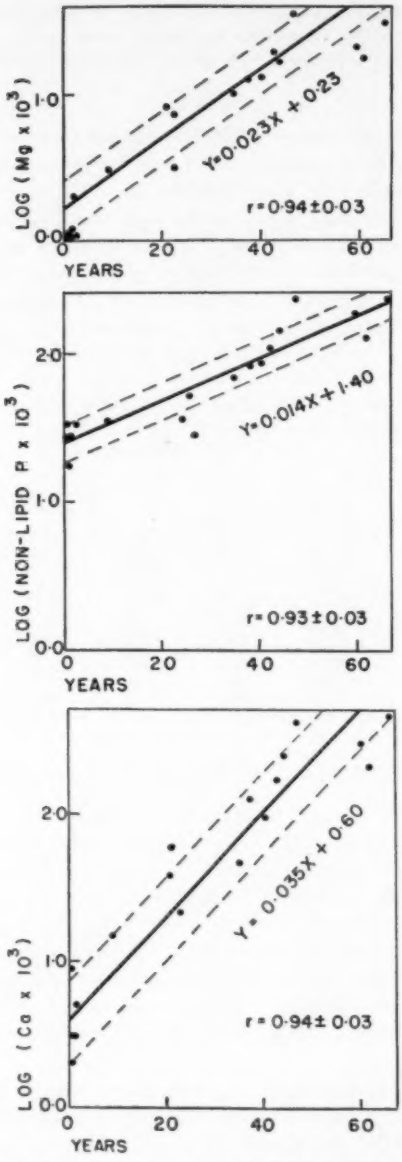

Chart 1 (grossly normal aortas).—Relation of the logarithm of the concentration of each of the minerals magnesium, nonlipid phosphorus and calcium to age. The solid line gives the calculated regression line, and the broken lines give the standard error of estimate of the logarithm of the concentration of the mineral from age.

lines the standard error of estimate of the logarithm of the mineral concentration derived from age alone. The concentration of calcium showed the greatest increase with aging, while that of phosphorus showed the least. The increase in the concentration of magnesium was intermediate in value. The relatively high concentration of nonlipid phosphorus in infancy is possibly related to the presence of phosphorus-containing proteins.

Since the age of the subjects in the grossly normal group (grade 0) was found to be significantly correlated with the thickness of intima ($r = 0.70 \pm 0.13$), it was important to determine whether the statistically significant correlation between the logarithm of the concentration of each of the minerals and age was related

TABLE 2.—*Normal Aortas—Coefficient of Correlation of the Mineral Concentration and of the Logarithm of Mineral Concentration with Age* *

	Coefficient of Correlation with Age					
	Mineral Concentration			Logarithm of Mineral Concentration		
	r.	S. E. r.	P	r.	S. E. r.	P
Nonlipid phosphorus	0.78	± 0.09	< 0.01	0.93	± 0.03	< 0.01
Calcium	0.78	± 0.09	< 0.01	0.94	± 0.03	< 0.01
Magnesium	0.83	± 0.08	< 0.01	0.94	± 0.03	< 0.01

* The mineral concentrations are given in table 1 in mg. per 100 mg. wet tissue. N = 17.

TABLE 3.—*Grossly Normal Aortas—Coefficients of Correlation and Partial Correlation with Age and with Intimal Thickness of the Logarithm of the Mineral Concentration and of the Concentrations of Fat-Free Dry Residue and Water* *

		Coefficient of Correlation with						Coefficient of Partial Correlation with	
		Age			Intimal Thickness			Age Excluding Intimal Thickness	Intimal Thickness Excluding Age
	N	r.	S. E. r.	P	r.	S. E. r.	P		
Logarithm phosphorus	17	0.96	±0.03	< 0.01	0.64	±0.15	< 0.01	0.87	0.00
Logarithm calcium	17	0.94	±0.03	< 0.01	0.75	±0.11	< 0.01	0.87	0.37
Logarithm magnesium	17	0.94	±0.03	< 0.01	0.76	±0.11	< 0.01	0.88	0.41
Fat-free dry residue	18	−0.12	±0.24	> 0.1	−0.44	±0.21	> 0.05	0.29	−0.50
Water	18	−0.10	±0.24	> 0.1	0.33	±0.22	> 0.1	−0.47	0.57

* All concentrations have been found as mg. per 100 mg. wet tissue (table 1).

to age per se or was simply determined by the association of age with this microscopic degree of atherosclerosis. Table 3 shows the coefficients of the correlation between the logarithm of mineral concentration and age, on the one hand, and the thickness of the intima on the other. The latter coefficients were considerably less than the former. On expressing these relationships as coefficients of partial correlation (shown in the last two columns of table 3) it is apparent that the association between mineral concentration and intimal thickness was of a secondary nature only, being dependent on the correlation between thickness of intima and age. When the effect of age was excluded, there was no correlation with thickness of intima. In this group of apparently normal aortas, the concentration of water and that of the fat-free dry residue were significantly correlated neither with age nor with thickness of intima.

Relation to Atherosclerosis.—As already seen in table 2 the concentrations of nonlipid phosphorus, calcium and magnesium increased significantly with the severity of the disease as judged by the gross grading. That of the fat-free dry residue did not increase significantly, while the concentration of water decreased. The concentrations of these substances were studied in relation to two other indexes of the degree of atherosclerosis, the thickness of intima and the concentration of

TABLE 4.—*All Aortas—Coefficient of Correlation of (a) the Concentration and (b) the Logarithm of the Concentration of Mineral, Fat-Free Dry Residue and Water with the Thickness of the Intima and the Concentration of Total Cholesterol*

		Coefficient of Correlation with					
		Intimal Thickness			Total Cholesterol		
	N	r.	S. E. r.	P	r.	S. E. r.	P
(a) Concentration *							
Nonlipid phosphorus	52	0.46	±0.11	< 0.01	0.55	±0.09	< 0.01
Calcium	52	0.44	±0.11	< 0.01	0.53	±0.10	< 0.01
Magnesium	52	0.34	±0.12	< 0.02	0.47	±0.10	< 0.01
Fat-free dry residue	53	−0.05	±0.14	> 0.1	0.04	±0.13	> 0.1
Water	50	−0.59	±0.09	< 0.01	−0.52	±0.10	< 0.01
(b) Logarithm of concentration							
Nonlipid phosphorus	52	0.65	±0.08	< 0.01	0.73	±0.06	< 0.01
Calcium	52	0.60	±0.09	< 0.01	0.69	±0.07	< 0.01
Magnesium	52	0.45	±0.11	< 0.01	0.67	±0.07	< 0.01

* All concentrations have been found as mg. per 100 mg. wet tissue (table 1).

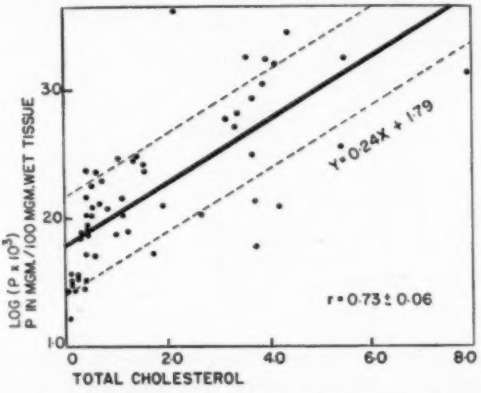

Chart 2 (all aortas).—Relation of the logarithm of the concentration of nonlipid phosphorus to the concentration of total cholesterol. The solid line gives the calculated regression line and the broken lines the standard error of estimate of the logarithm of mineral concentration from total cholesterol concentration.

total cholesterol. It has previously been suggested that the concentration of total cholesterol might serve as a chemical index of the severity of atherosclerosis.[1]

Table 4 shows the linear and the logarithmic relationship between the concentrations of the minerals and both the thickness of the intima and the concentration of total cholesterol. As was found for age, the logarithm of the concentration of each mineral was more highly correlated with the severity of atherosclerosis than was the concentration itself. The values for the highest correlation, that

between the logarithm of the concentration of nonlipid phosphorus and the concentration of total cholesterol, are plotted in chart 2. Again, the concentration of water was significantly decreased and that of the fat-free dry residue was not changed in relation to either of these indexes.

In order to determine the relation of the concentrations of the minerals, the water and the fat-free dry residue to the degree of atherosclerosis with the age factor entirely eliminated, the difference between the concentration of the substance in the most diseased portion of each individual aorta and that in either the normal or the least diseased portion of the same aorta was determined. Table 5 shows the mean of these differences, the standard error of the mean, and the *P* value indicating whether the mean difference is significantly different from zero.

TABLE 5.—*Mean Difference in the Concentration of Nonlipid Phosphorus, Calcium, Magnesium, Fat-Free Dry Residue and Water Between the Normal or Least Diseased and the Most Severely Diseased Portions of Individual Aortas* *

	N	Mean Difference	S. E. M.	P
Nonlipid phosphorus	16	0.85	±0.29	< 0.01
Calcium	16	2.20	±0.80	< 0.02
Magnesium	16	0.026	±0.012	> 0.05
Fat-free dry residue	17	1.32	±1.82	> 0.1
Water	15	−6.40	±2.15	< 0.01

* Concentrations have been found as mg. per 100 mg. wet tissue (table 1).

TABLE 6.—*All Aortas—Intercorrelation of the Concentrations of Nonlipid Phosphorus, Calcium and Magnesium in the Aortas Arranged in Groups According to the Degree of Atherosclerosis* *

		Coefficient of Correlation of								
		Phosphorus with Calcium			Phosphorus with Magnesium			Calcium with Magnesium		
Gross Grade	N	r.	S. E. r.	P	r.	S. E. r.	P	r.	S. E. r.	P
0	15	0.99	± 0.00	< 0.01	0.98	± 0.01	< 0.01	0.97	± 0.02	< 0.01
1	10	0.94	± 0.04	< 0.01	0.96	± 0.02	< 0.01	0.73	± 0.16	< 0.02
2	14	0.999	± 0.00	< 0.01	0.93	± 0.04	< 0.01	0.92	± 0.04	< 0.01
3	13	0.98	± 0.04	< 0.01	0.84	± 0.09	< 0.01	0.84	± 0.09	< 0.01
All aortas	52	0.95	± 0.01	< 0.01	0.86	± 0.04	< 0.01	0.86	± 0.04	< 0.01

* Concentrations have been found as mg. per 100 mg. wet tissue (table 1).

The concentrations of nonlipid phosphorus, calcium and magnesium were significantly increased in the more diseased tissue, while that of water was decreased. The concentration of fat-free dry residue showed no significant change. However, the standard error of the mean difference was large, especially for calcium, and the significance of the increase in the concentrations of calcium and magnesium was not nearly as great as that previously reported for cholesterol, lecithin or sphingomyelin.[1] In fact, it was found that in many of the aortas the concentrations of the minerals were actually higher in the less severely diseased portions.

Relation Between Concentrations of Phosphorus, Calcium and Magnesium.—An increase in the concentration of one mineral was accompanied by a proportionate increase in the concentrations of the other two. In table 6 the correlation of each mineral with the other two is shown for each of the four grades. Some

of the correlations, particularly that between the concentrations of phosphorus and calcium, are almost perfect. The highest coefficients of correlation were for grade 0 and the lowest were for grade 3. The more severe the atherosclerosis the less significant was the correlation between the concentrations of the minerals. Chart 3 depicts the relation between the concentration of nonlipid phosphorus and that of calcium. This relation is given by the regression equation

$$P = 0.36\ Ca + 0.07$$

where P is the concentration of nonlipid phosphorus and Ca is the concentration of calcium in mg. per 100 mg. fresh tissue. On a molecular basis the ratio of the concentration of calcium to that of nonlipid phosphorus is approximately 2:1.

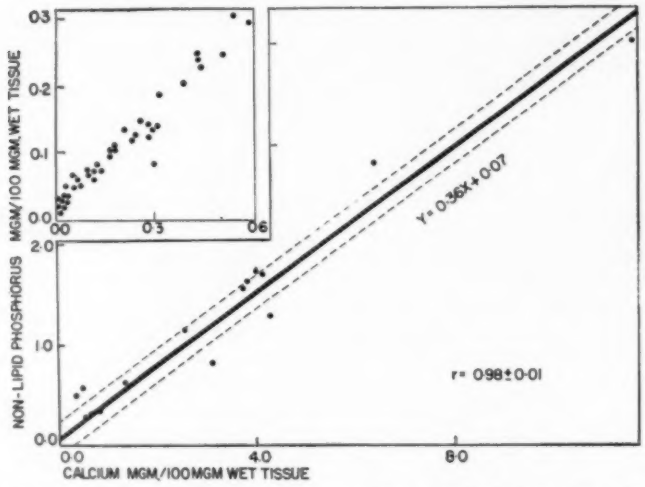

Chart 3 (all aortas).—Correlation of the concentration of nonlipid phosphorus with the concentration of calcium. The solid line gives the calculated regression line and the broken lines the standard error of estimate of the concentration of nonlipid phosphorus from the calcium concentration. The insert shows the correlation at lower concentrations of calcium and nonlipid phosphorus.

COMMENT

Previous workers who have been concerned with the relation of the mineral content of the aorta to age have, to a great extent, relied on histological methods. However, studies on the calcification of blood vessels in which the sections were stained with hematoxylin or alum hematoxylin are open to question. In a comprehensive review of the staining reactions for calcium, Cameron [13] pointed out that hematoxylin does not stain calcium salts. The reaction obtained with hematoxylin in calcified tissues depends in part on the presence of minute amounts of iron and mostly on the ground substance appearing at the site of pathological calcification.

Using microincineration methods, Ravault [4b] and Blumenthal, Lansing and Wheeler [4c] reported an increase of minerals in the media of normal-appearing aortas

13. Cameron, G. R.: J. Path. & Bact. **33**:929, 1930.

with advancing age. By chemical analysis, Weinhouse and Hirsch [4d] found that in the media, separated from intima (which in many cases was atherosclerotic), the calcium content increased with aging. In the present investigation it has been found that the logarithm of the concentration of nonlipid phosphorus, calcium or magnesium in the normal-appearing aorta is highly correlated with age. The fact that the intima of the normal vessel of the present study represented only a small fraction of the total bulk of the specimen, taken in conjunction with the above findings, suggests that the minerals associated with increasing age are deposited in the media. The effect of aging would, therefore, appear to be (1) a relatively small increase in the concentrations of certain lipids (particularly ester cholesterol and sphingomyelin) and (2) a relatively great increase in the concentrations of nonlipid phosphorus, calcium and magnesium.

The changes in the media of the aorta associated with aging are similar in nature to those characteristic of the condition of Mönckeberg's sclerosis in the vessels of the limbs. In the aorta the situation is complicated by the presence of superadded intimal atherosclerosis. In arteries affected by Mönckeberg's sclerosis, however, atherosclerotic changes in the intima are usually slight as compared with the characteristic morbid changes in the media.

The logarithmic nature of the increase in the concentrations of minerals (in contrast to the linear increase in the concentrations of the lipids) may possibly be related to the fact that they are deposited in crystalline form. The use of roentgen ray diffraction technics has revealed the occurrence of the crystalline substance, carbonate-apatite, in bone and in pathological calcification. Frondel and Prien [3f] have found carbonate-apatite in the calcified aorta. The logarithmic rate of increase in the concentrations of minerals might be explained by the crystals growing from minerals in solution in the interstitial fluid of the media of the vessel. The concentrations of minerals in the blood serum and, therefore, in the interstitial fluid, might be presumed to be fairly constant. Under these circumstances the rate of growth of crystals, i.e, the rate of increase in the concentrations of the minerals in the media, would be a function of the surface area of the crystals and, on theoretical grounds, would be expected to be nonlinear.

The relation of the mineral content of the media to the degree of overlying intimal disease has been the subject of considerable difference of opinon. Policard, Morel and Ravault [4c] found that the concentration of calcium and particularly the concentration of magnesium were diminished in those parts of the media under areas of atheromatous intima. Blumenthal, Lansing and Wheeler,[4e] on the other hand, reported that minerals were increased in the media and that atheromatous changes did not appear in the intima until after the appearance of mineral in the media. Farkas and Fasal,[6] using hematoxylin and eosin staining, found no relation between atherosclerosis of the intima and the intensity of the hematoxylin staining in the media. It would appear from the results of the present investigation that there can be considerable mineral salt infiltration of the media independent of any overlying intimal disease. Even in the presence of extensive atherosclerosis of the intima the increase of the concentration of mineral in the vessel is, except in a few cases, no higher than would be expected to result from the process of aging alone. In these few cases the intima itself may have become calcified and thus have contributed to the high mineral concentration.

The relative concentrations of calcium and phosphorus were investigated by Baldauf,[3a] Wells,[3b,c] and Schoenheimer.[3e] Each of these workers found that the ratio of calcium to phosphorus concentrations closely approximated that of bone. They concluded that pathological calcification and normal ossification were essentially similar. Wells,[3d] in fact, mentioned that true bone formation had occurred in the aorta. In the present study, increments of calcium were accompanied by proportional increments of phosphorus in the ratio of 1 : 0.36. This ratio is constant, despite great variations in age and in the degree of atherosclerosis associated with a thousand-fold increase in the concentration of calcium. This agrees fairly well with the ratio of 1 : 0.40 found by Schoenheimer [3e] in two calcified portions of aorta.

SUMMARY

The concentrations of nonlipid phosphorus, calcium, magnesium, water and fat-free dry residue were determined in a series of normal and atherosclerotic human aortas.

A statistical analysis of the results was carried out in relation to aging and to the degree of atherosclerosis.

In apparently normal aortas the result of aging, apart from atherosclerosis, was an increased concentration of each of the minerals. The rate of increase was, in each case, logarithmic.

Although the concentration of each of the minerals in atherosclerotic portions of aorta was significantly greater than that in normal portions, the increase associated with atherosclerosis was small in comparison with that associated with aging. The concentration of water was significantly decreased in relation to the degree of atherosclerosis, while that of the fat-free dry residue was unchanged.

The relative proportion in which the minerals are deposited was found to be constant, suggesting the possibility that they are in chemical combination. The ratio of calcium to phosphorus was similar to that found in bone.

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Dr. J. C. Paterson contributed advice on many aspects of this problem. The pathological material was largely obtained from the Laboratory Service, Westminster Hospital, Department of Veterans Affairs, London, Ont., Canada.

THE LIPOPROTEIN OF GAUCHER'S DISEASE

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DURING the past three decades the interest of investigators working on Gaucher's disease from the point of view of chemical pathology has been focused mainly on the evaluation of the lipids extracted from various organs of patients with this disease. The discovery that abnormally large amounts of a cerebroside—kerasin [1] or its glucose analog [2]— were present in lipid extracts of the spleen appeared as an encouraging forerunner to the hope of eventually clarifying the nature of the metabolic defect that gives rise to the Gaucher cell. It was justifiably assumed that the large amount of kerasin isolated from whole organs actually reflected a major fraction of the lipid material that accumulated in the individual cells. The use of the periodic acid-fuchsin stain of Morrison and Hack,[3] which purports to stain the cerebroside within Gaucher cells by reacting with the hexose moiety of the cerebroside, has further strengthened this assumption. Yet certain well known observations militate against the acceptance of kerasin per se as the sole abnormal chemical feature of the Gaucher cell: (*a*) The drastic treatment to which the tissue is subjected in isolating kerasin (boiling organic solvents) raises the question whether kerasin may not represent the lipid component of a much larger intracellular biological and chemical entity; (*b*) though kerasin is freely soluble at room temperature in chloroform-methanol, dehydrated tissue sections treated with these organic solvents will not lose their kerasin; (*c*) pure kerasin itself has no specific absorption in the ultraviolet spectral range, yet Gaucher cells show a diffuse absorption around 2,800 angstroms; (*d*) the kerasin phagocytosed by the reticulum cells, in lipophagia experimentally induced by injecting pure

An abstract of part of this work was presented at the thirty-fifth annual meeting of the American Society for Experimental Pathology, Atlantic City, N. J., April 20, 1950.

From the Department of Pathology, Harvard Medical School, Division of Laboratories and Research, The Children's Medical Center, and The Children's Cancer Research Foundation.

1. (*a*) Lieb, H.: Ztschr. f. physiol. Chem. **140**:305, 1924; (*b*) **170**:60, 1927. (*c*) Lieb, H., and Mladenović, M.: Ibid. **181**:208, 1929. (*d*) Lieb, H.: Ibid. **271**:211, 1941. (*e*) Epstein, E., and Lorenz, K.: Ibid. **192**:145, 1930. (*f*) Epstein, E.: Biochem. Ztschr. **145**:398, 1924. (*g*) Kaye, I. A.: J. Lab. & Clin. Med. **25**:1117, 1940.

2. (*a*) Aghion, H.: La maladie de Gaucher dans l'enfance (forme cardiorénale), Thesis, Paris, 1934, pp. 1-98. (*b*) Danielson, I. S.; Hall, C. H., and Everett, M. R.: Proc. Soc. Exper. Biol. & Med. **49**:569, 1942. (*c*) Halliday, N.; Deuel, H. J., Jr.; Tragerman, L. J., and Ward, W. E.: J. Biol. Chem. **132**:171, 1940. (*d*) Klenk, E.: Ztschr. f. physiol. Chem. **267**:128, 1940. (*e*) Klenk, E., and Rennkamp, F.: Ibid. **272**:280, 1942; (*f*) **273**:253, 1942. (*g*) Klenk, E.: Ibid. **153**:74, 1926.

3. Morrison, R. W., and Hack, M. H.: Am. J. Path. **25**:597, 1949.

kerasin intravenously into animals is easily extracted from these cells with organic solvents, whereas the kerasin of the Gaucher cells is not.[3]

These points have evoked in the minds of many investigators the thought that the kerasin in the Gaucher cell may be "bound," probably to some normal constituent of the cell. Since the cellular protein is the constituent most likely to be affected by the procedures commonly employed in the isolation of the cerebroside, one envisaged the kerasin in the Gaucher cell to be present as a kerasin-protein complex which would split to yield the free cerebroside only after denaturation, or drastic alteration in the spatial relationships of the protein moiety, had taken place.

The present paper describes the isolation and properties of a lipoprotein fraction which was isolated from two spleens removed from patients with Gaucher's disease and which had kerasin as the sole lipid moiety of the complex. In view of the fact that this fraction accounts for the major part of the so-called abnormal lipid that chemically characterizes Gaucher cells, and because of its rather special

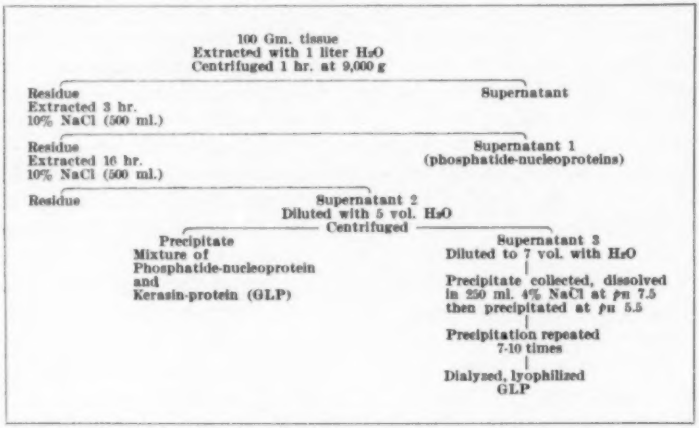

Chart 1.—Procedure for the isolation of the lipoprotein of Gaucher's disease.

properties, one feels justified in designating this fraction the lipoprotein of Gaucher's disease.

ISOLATION OF THE LIPOPROTEIN

Two spleens (weighing 650 Gm. and 580 Gm., respectively) obtained at operation from patients with Gaucher's disease (7 and 20 years old, respectively) were frozen immediately in dry ice. Just before being processed, the two organs were allowed to thaw at room temperature until they were soft enough to permit cutting with a knife. From this point on the fractionation was carried out in a cold room operating at a temperature of 2 C. A schematic outline of the procedure employed is depicted in chart 1. Batches of 100 Gm. of each spleen were minced and extracted in portions of 50 Gm. with 1 liter of cold distilled water in a Waring blendor* for two minutes. The resulting suspension was centrifuged for one hour at 9,000 g., and the residue was extracted for three hours with 500 ml. of cold 10 per cent sodium chloride solution with moderate stirring. At the end of three hours, the first saline extract (supernatant 1) was separated by centrifugation at 9,000 g. for one hour. The residue then was again extracted with a 10 per cent solution of sodium chloride (500 ml.) for 16 hours. Centrifugation at 9,000 g. for one hour yielded a viscous pink supernatant (supernatant 2). The final residue, weighing 1.7 Gm. (dry), was discarded. The second saline supernatant was diluted with five volumes

of cold distilled water, whereupon there was an immediate development of turbidity. The diluted extract was allowed to stand 24 hours in the cold. The white precipitate that collected was centrifuged off, and the supernatant (supernatant 3) was further diluted to seven volumes of the original amount by the slow addition of distilled water. This second dilution appeared to be critical, since the addition of water in excess caused a pink splenic chromogen to precipitate with the lipoprotein fraction. In such cases, numerous salt fractionations and isoelectric precipitation steps have not removed this contaminant. The white flocculate which appeared on dilution of supernatant 3 was allowed to settle in the cold overnight and was centrifuged off. The precipitate then was taken up in 250 ml. of 4 per cent sodium chloride solution adjusted to p_H 7.5 with alkali and reprecipitated at p_H 5.5 with dilute hydrochloric acid. The precipitation was repeated seven to 10 times, until lyophilized aliquots were phosphorus free. Since pilot experiments indicated a considerable change in the solubility of the lipoprotein fraction following lyophilization, only an aliquot of the final fraction in a 4 per cent saline solution was neutralized, dialyzed against distilled water for 48 hours in the cold and lyophilized. The white fluffy powder (Gaucher lipoprotein) thus obtained was used for the analyses, while the major part of the lipoprotein fraction was kept in a fully hydrated state in saline solution and used for determination of osmotic pressure, organic anion-binding power and solubility studies, as will be described later.

Supernatant 1 on acidifying to p_H 5.0 yielded a bulky white precipitate which was centrifuged off, redissolved in dilute alkali, reprecipitated by the addition of acid, dialyzed and lyophilized. The lipid moiety was extracted from the dry material by boiling it with chloroform-methanol (2:1) for two hours; this accounted for 32 per cent of the total dry powder. It contained 2.8 per cent phosphorus and 4 per cent galactose. The residue from the lipid extraction contained 1.5 per cent phosphorus and gave a strong violet color with Dische's diphenylamine test for desoxyribose nucleic acid.[4] This indicated that supernatant 1 contained a mixture of most of the splenic liponucleoproteins and some of the Gaucher lipoprotein.

The precipitate obtained by fivefold dilution of supernatant 2, after dialysis and lyophilization, yielded a grayish white powder containing 42 to 45 per cent lipid with 1.7 per cent lipid phosphorus and 8.6 to 12.8 per cent lipid galactose in the six different preparations that were analyzed. The protein-phosphorus was 1.0 per cent. Thus the precipitated fraction from supernatant 2 appeared to consist of a mixture of tissue liponucleoproteins greatly enriched with the Gaucher lipoprotein. This was borne out when this fraction was dissolved in a 2 per cent saline solution at p_H 3.0 and precipitated at p_H 4.5. The precipitate was collected and redissolved and reprecipitated in the same manner five times. After dialysis and lyophilization, this procedure yielded a fluffy white powder identical in chemical composition with the Gaucher lipoprotein obtained from supernatant 3.

The total yield of Gaucher lipoprotein from the two spleens was estimated as 3.8 per cent and 4.2 per cent of the fresh weight, respectively, and accounted for about 70 per cent of the total kerasin present in the organs.

CHEMICAL COMPOSITION OF THE LIPOPROTEIN

On attempting to separate the lipid and protein moieties of the lyophilized lipoprotein fraction, it was found necessary to extract the dry material for 6½ hours with a boiling mixture of chloroform-methanol (2:1) before complete extraction of the lipid moiety could be effected. The lipid content of the Gaucher lipoprotein was found to be 62 per cent and 63.8 per cent for the fractions obtained from the two spleens. Analyses of the lipid moieties of the lipoprotein fraction gave the following results: 1.9 per cent nitrogen, no phosphorus, 19.6 to 21.4 per cent galactose by the orcinol method [5] and no free amino nitrogen. After acid hydrolysis,

4. Dische, Z.: Mikrochemie **7**:33, 1929.

5. Brückner, J.: (*a*) Ztschr. f. physiol. Chem. **268**:163, 1941; (*b*) **275**:73, 1942.

the amino nitrogen value was 1.87 per cent. Baryta hydrolysis of the lipid moiety according to Klenk [2g] yielded the crystalline sulfate ester of sphingosyl-galactose containing 2.78 per cent nitrogen, 2.72 per cent amino nitrogen and 33.7 per cent galactose. The fatty acid component was isolated as the methyl ester melting at 57 C., which corresponds to the value for the ester of lignoceric acid.[6] The free acid from the baryta hydrolysate melted at 84 C. (copper block). The analytic data presented here indicated that the lipid moiety of the lipoprotein complex was constituted solely by the cerebroside kerasin and thus accounted for more than 70 per cent of the kerasin present in the fresh organs. The identity of the hexose as galactose was confirmed by the fact that the values obtained in parallel runs by the reductimetric method,[7] orcinol method [5a] and Dische's [4] phloroglucinol method agreed closely. If glucose had been present, a wide discrepancy between the values obtained by the reductimetric method, on one hand, and the orcinol method and the phloroglucinol reaction, on the other, would have been expected, since, as observed by Brückner [5a] and Dische [4] and confirmed in the present investigation, the intensity of color developed by orcinol and phloroglucinol with glucose is about 70 per cent less than that developed with galactose, while the reducing value of glucose is higher than that of galactose.

The protein moiety constituted 38 per cent of the lipoprotein fraction. It was found to contain 14.7 per cent total nitrogen, 1.2 per cent amide nitrogen and no phosphorus.

The protein moieties from three separate preparations of Gaucher lipoprotein were hydrolyzed with 6 times normal hydrochloric acid under nitrogen in sealed tubes for 20 hours at 110 C. The acid was removed by taking the samples to dryness *in vacuo*, adding water and taking to dryness again. This process was repeated three times. Finally, the samples were made up to suitable volume, the insoluble humin was centrifuged off, and suitable aliquots were taken in order (*a*) to identify the amino acids present in the hydrolysate with one-dimensional and two-dimensional paper chromatography and (*b*) to determine quantitatively some of the amino acids by appropriate colorimetric methods. Aspartic and glutamic acid were isolated from the hydrolysates as barium salts by Foreman precipitation. These salts were converted into the free amino acids by adding the exact amount of dilute sulfuric acid, and then the copper salts of aspartic and glutamic acid were formed by boiling with an excess of copper carbonate. The precipitated copper aspartate and copper glutamate were dehydrated with cold acetone. The water-soluble copper glutamate was separated from the insoluble copper aspartate. Aspartic and glutamic acid was then estimated by the nitrogen content of each fraction after the copper had been precipitated with hydrogen sulfide. Arginine was determined by a modification of the method of Dubnoff and Borsook,[8] using the Sakaguchi reaction. Methionine was estimated by a micro-adaptation of the nitroprusside method of McCarthy and Sullivan.[9] Tyrosine was determined by the Millon-Nasse reaction. Alkaline hydrolysis according to Lugg [10] was employed in estimating tryptophan, and the values thus obtained agreed with those obtained on unhydrolyzed samples of the protein moieties by a modification of the method of May and Rose.[11] Semiquantitative estimation of other amino acids was effected by elution of spots from the chromatograms and by measuring the color produced by each amino

6. Rosenheim, O.: Biochem. J. **7**:604, 1913; **8**:110, 1914.
7. Hanes, C. S.: Biochem. J. **23**:99, 1929.
8. Dubnoff, J. W., and Borsook, H.: J. Biol. Chem. **138**:381, 1941.
9. McCarthy, T. E., and Sullivan, M. X.: J. Biol. Chem. **141**:871, 1941.
10. Lugg, J. W. H.: Biochem. J. **31**:1422, 1937.
11. May, C. E., and Rose, E. R.: J. Biol. Chem. **54**:213, 1922.

acid with 1 per cent ninhydrin in butanol. The amino acid composition of the protein moieties of the three different lipoprotein preparations agreed very closely. The average values are shown in the table.

It is evident that the protein moiety of the lipoprotein complex presents no significant feature in its amino acid composition except for the absence of valine.

SOLUBILITY AND AVERAGE PARTICLE SIZE

In the course of the isolation of the Gaucher lipoprotein, it was noticed that the lipoprotein underwent considerable change in solubility after it had been lyophilized or dried *in vacuo* at room temperature. The lipoprotein fraction precipitated from supernatant 3 was found to be soluble in a 4 per cent solution of sodium chloride adjusted to p_H 7.5 and almost completely insoluble in the same salt concentration at p_H 4.2. The solubility at neutral p_H increased with increasing salt concentration, reaching a maximum (2.2 per cent) at 10 per cent salt concentration. The solubility decreased sharply with decreasing salt concentration, ranging from 1 per cent lipoprotein concentration in 4 per cent saline to 0.009 per cent in distilled water (dialysis against distilled water) as measured by the nitrogen content of the supernatant fluid after centrifuging off the solid phase. The lipoprotein was found to be more soluble in alkaline

Amino Acid Content of Protein Moiety

Aspartic acid	5.4	%	} Ba-salt,
Glutamic acid	17.2		} Cu-salt
Serine	2.9		.....
Glycine	8.0		.....
Cystine	1.9		.....
Tyrosine	5.7		Millon
Alanine	9.2		.....
Leucine (isoleucine)	15.5		.....
Lysine	4.0		.....
Arginine	11.2		Sakaguchi
Methionine	2.4		Nitroprusside
Tryptophan	1.36		May-Rose and Lugg
Proline	4.2		.....
Phenylalanine	2.9		.....
	92.66		

solutions (p_H 7.5 to p_H 8.5) with an ionic strength below 0.5. However, at p_H levels higher than 9.5, 1 per cent solutions of the lipoprotein produced thixotropic gels. Dehydration of the lipoprotein fraction, either by lyophilization or over calcium chloride *in vacuo* at room temperature, rendered the preparations insoluble in salt solutions containing less than 10 per cent salt concentration, and 0.2 per cent solutions of the material could be prepared only in 8 per cent sodium chloride solution adjusted with alkali to p_H 8.5.

Because of the technical difficulties arising from the limited conditions under which true solutions of the lipoprotein could be prepared, determination of molecular weight by osmometry involved the use of rather dilute lipoprotein solutions, a fact which detracts somewhat from the accuracy obtainable with this method. An aliquot of the lipoprotein fraction obtained from supernatant 3 was dissolved in 4 per cent saline solution at p_H 7.5 and precipitated by the addition of ½ volume of saturated ammonium sulfate and dialyzed in the cold against large volumes of 0.5 M phosphate buffer at p_H 6.5 until no NH_4 ions could be detected in the dialyzate. The contents of the dialysis bag were then centrifuged, and after removing the undissolved portion of the lipoprotein, the concentration in the supernatant was estimated by its nitrogen content. Dilutions of the supernatant to give 0.6 per cent, 0.4 per cent and 0.2 per cent solutions of the lipoprotein were then made with the same phosphate buffer, and the osmotic pressure for each concentration was determined with a modified Zimm osmometer,[12] previously calibrated with sucrose and crystalline bovine albumin. Extrapolation to infinite dilution of the $(\pi/C,C)$ curve indicated a number average molecular weight of about 320,000 for the lipoprotein. Corrections for the Donnan effect were deemed unnecessary in view of the low protein concentrations used.

12. The all-metal apparatus with a 3.6 ml. capacity was constructed by Mr. Fred Savage.

STABILITY OF THE LIPID-PROTEIN BOND

The cerebroside moiety of the isolated lipoprotein fraction appeared to be firmly bound to the protein. Six and a half hours of boiling of the lyophilized lipoprotein with a chloroform-methanol (2:1) mixture was necessary before the kerasin could be completely extracted. However, if the lipoprotein was in a partially hydrated state, the same solvent mixture at 50 C. effected complete extraction of the kerasin in two hours. Thus, 50 mg. of the lipoprotein, precipitated out from a saline solution by dialysis against distilled water, was allowed to dry at room temperature in a desiccator until the precipitate contained about 6 per cent water (final weight, 53 mg.). This was then extracted for two hours at 50 C. with 10 ml. of chloroform-methanol (2:1) in a loosely sealed vessel. At the end of this period, the precipitated denatured protein was centrifuged off and washed once with chloroform-methanol. The washing was added to the supernatant. Evaporation and drying *in vacuo* of the supernatant yielded 30.8 mg. of kerasin. Further treatment of the precipitate with boiling chloroform-methanol did not extract any more lipid.

Since the degree to which the lipid moiety of the Gaucher lipoprotein resisted extraction with organic solvents appeared to be determined by the state of hydration of the lipoprotein, it seemed probable that if the protein moiety were denatured by means other than boiling organic solvents, the splitting off of free kerasin would result. Accordingly, 50 mg. of lyophilized Gaucher lipoprotein (containing 62 per cent cerebroside) was ground in 10 ml. of eight times molar guanidine-hydrochloride at room temperature. The resulting clear solution became turbid, and on standing in the cold for fifteen minutes it showed gradual development of a heavy white flocculate. After this had been allowed to settle for 24 hours in the cold, it was centrifuged off, and the precipitate was washed once with eight times molar guanidine-hydrochloride, dialyzed against running tap water and dried *in vacuo*. The dried material weighed 26.7 mg. and proved to be kerasin. Chromatographic examination of the acid hydrolysate of this precipitate showed it to be free of traces of protein, since no amino acids were detectable.

The combined clear supernatants obtained after the lipid precipitate had been centrifuged off were dialyzed with repeated changes of distilled water to remove all the guanidine-hydrochloride. After three days of dialysis in the cold, the denatured protein that had precipitated out was centrifuged off and dried *in vacuo*. This yielded 18.2 mg. of brown material which, on extraction with boiling chloroform-methanol, yielded less than 3 per cent lipid.

ORGANIC ANION-BINDING CAPACITY

These experiments were undertaken with the object of obtaining information regarding the disposition of acid-binding loci on the surface of the lipoprotein molecule. The dialysis equilibrium procedure described by Klotz and his associates[13] was followed. Crystalline bovine albumin was always run in a parallel experiment. The spectral curves were studied after 24 and 48 hours of equilibration at 2 C. No measurable increment of binding of methyl orange or Biebrich scarlet occurred after 24 hours of equilibration.

The results of the experiments with methyl orange[14] are given in chart 2. The binding powers of equal amounts (54.8 mg.) of bovine albumin and Gaucher lipoprotein dissolved in 10 ml. of molar sodium chloride solution adjusted to p_H 6.5 were compared. The dialyzing fluid consisted of 40 ml. of molar sodium chloride solution with a 2.3×10^{-5} molar concentration of methyl orange. The dye was found to be monomeric in the salt concentration used, the curve showing no shifts from that obtained with the same dye concentration in 0.1 M phosphate buffer at p_H 6.5. The lipoprotein bound more of the dye (1.02×10^{-5} M) than did the bovine albumin (0.86×10^{-5} M), in spite of the fact that 62 per cent of the lipoprotein consisted of inert lipid. This interesting observation raised the question whether the ratio of bovine albumin-bound dye to lipoprotein-bound dye would be reversed if a larger organic anion were used as a coating for the available surface groups of the lipoprotein and bovine albumin molecules. The choice of Biebrich scarlet (C. I. 280)[14] indicated itself by virtue of the fact that while providing a structural analogy with methyl orange, it also offered the desired increment in molecular size (a naphthalene ring) plus an additional acidic group (compare

13. Klotz, I. M.; Walker, F. M., and Pivan, R. B.: J. Am. Chem. Soc. **68**:1486, 1946.

14. The reagent grade product of the National Aniline Division of the Allied Chemical and Dye Corporation was used.

formulas in charts 2 and 3). However, since Biebrich scarlet was found to be salted out in molar sodium chloride solution, the dialysis equilibrium experiments were carried out in 0.4 M phosphate buffer at p_H 6.5. In this salt concentration the dye was found to be monomeric, the spectral curve exhibiting no beta and gamma shifts.

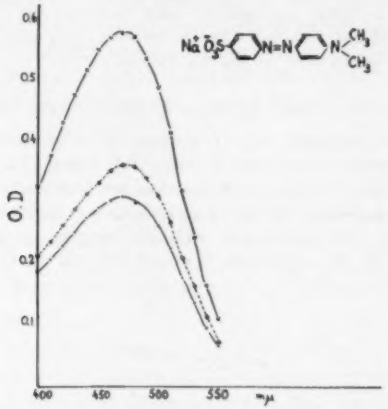

Chart 2.—Spectral curves of residual dye showing the extent of binding of methyl orange by equal amounts of crystalline bovine albumin (o---o---o) and Gaucher lipoprotein (.——.——.) as measured by the residual dye concentrations outside the dialysis bags. It is evident that the Gaucher lipoprotein (containing 62 per cent inert lipid) binds more organic anion than does an equal amount of bovine albumin. The methyl orange concentration in the control (x——x——x) was 2.3×10^{-5} molar in molar sodium chloride solution adjusted to p_H 6.5.

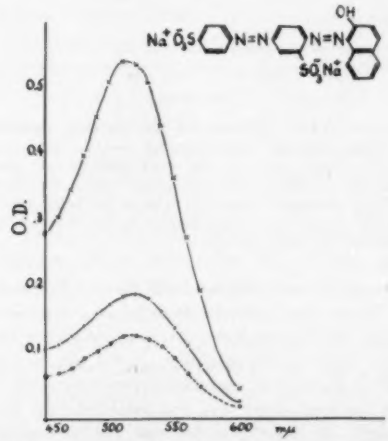

Chart 3.—Dialysis equilibrium experiments with Biebrich scarlet. The residual dye concentrations indicate a greater degree of binding of Biebrich scarlet by bovine albumin (o---o---o) as compared with the binding capacity of an equal amount of Gaucher lipoprotein (.——.——.). The Biebrich scarlet concentration in the control (x——x——x) was 1.8×10^{-5} molar Biebrich scarlet in 0.4 M phosphate buffer at p_H 6.5.

Chart 3 shows the result of such an experiment. The dialyzing buffer solution contained 1.8×10^{-5} molar concentration of Biebrich scarlet. The dialysis bags contained 80 mg.

of bovine albumin and 80 mg. of Gaucher lipoprotein, respectively. It is evident that under the conditions employed the amount of this dye bound by bovine albumin (1.38×10^{-5} molar) is greater than that fixed by an equal amount of lipoprotein (1.17×10^{-6} molar).

As a sequel to this experiment, the loss of protein-bound dye with prolonged dialysis against repeated changes of buffer was studied. After four days the bovine albumin had lost all the Bierbrich scarlet it had adsorbed, whereas the lipoprotein retained 15 per cent of the original amount of dye adsorbed after six days of dialysis.

RESISTANCE TO THE ACTION OF PROTEOLYTIC ENZYMES

Because the large lipid component might be expected to confer on the Gaucher lipoprotein a certain amount of resistance to enzymatic hydrolysis, the extent to which crystalline trypsin (Worthington), pepsin (Merck), papain and dog-liver cathepsin might act on the lipoprotein was investigated. The lipoprotein (50 mg. in each case) was incubated at 37 C. with 0.003 per cent pepsin in 0.05 N hydrochloric acid, with 0.04 per cent trypsin in 0.1 M phosphate buffer at p_H 7.8, with 0.008 per cent papain in citrate buffer at p_H 4.5 and with a dog-liver homogenate glycerol extract at p_H 7.0. In each case an equal amount (calculated for protein

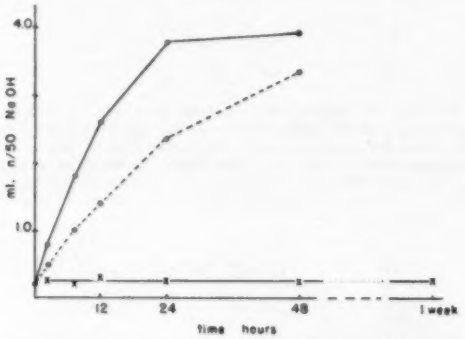

Chart 4.—Graph illustrating the resistance of the Gaucher lipoprotein to the proteolytic action of pepsin. Fifty milligrams of lipoprotein (x———x) was incubated with 0.003 per cent pepsin in 0.05 N hydrochloric acid for one week without any measurable hydrolysis, as determined by formaldehyde titration. On the other hand, the denatured protein moiety (o---o) of the lipoprotein underwent proteolysis almost as rapidly as heat-denatured bovine alumin (o——o), suggesting that the resistance displayed by the intact lipoprotein was probably due to the protective effect of the lipid moiety.

moiety of Gaucher lipoprotein) of heat-denatured (83 C. for 10 minutes) bovine albumin was incubated as a control. At various intervals, aliquots were neutralized, and the degree of hydrolysis was determined by formaldehyde titration. While bovine albumin underwent maximal proteolysis within 36 hours of incubation in the case of each enzyme tested, the lipoprotein proved completely resistant to enzymatic action after being incubated for one week. On the other hand, when the lipid moiety was first extracted from the lipoprotein by boiling the lipoprotein for 6½ hours with chloroform-methanol, and the denatured protein moiety was subjected to the action of the enzymes listed previously, maximal hydrolytic action was observed within 48 hours of incubation. An illustrative experiment showing the resistance to enzymatic hydrolysis displayed by the intact lipoprotein is depicted in chart 4.

It would appear, therefore, that the resistance to proteolytic enzymes displayed by the intact lipoprotein is due to the large cerebroside component of the fraction rather than to any structural characteristic of the protein moiety.

COMMENT

The apparent obscurity to which the study of cellular proteins in Gaucher's disease has hitherto been consigned is probably the result of two main difficulties that have faced investigators in this field. One of these has been the unsuitability of the available material in terms of protein fractionation work, either because it had been obtained several hours post mortem or because it had already been treated with formaldehyde solution or other fixatives. The second factor is the paucity of present day information on the nature, the properties and reproducible methods of fractionation of tissue proteins.

The fact that, as presented here, kerasin has been isolated in the form of a lipoprotein from two spleens from persons with Gaucher's disease by a relatively mild procedure and without the use of organic solvents can be regarded as adequate evidence that the cerebroside in the Gaucher cell is present in the form of a lipoprotein. The stability of the lipid-protein bond in the isolated lipoprotein fraction accounts for the difficulty that is experienced in trying to extract kerasin from tissue sections with organic solvents. It is evident from data presented here that the denaturation of the protein moiety, either with boiling organic solvents or with eight times molar guanidine-hydrochloride at room temperature, is necessary before the kerasin will be split free from the lipoprotein complex and thus become soluble in chloroform, methanol or alcohol. The fact that the lipoprotein fraction has a single lipid component and a large average molecular weight, that it presents special solubility characteristics, that it can be taken through numerous salt and isoelectric precipitations without change in composition and that the lipid-protein bond has remarkable tenacity can be accepted, without fear of serious contradiction, as indicative of a biochemical entity. The organic ion-binding power of the lipoprotein fraction is of a magnitude to warrant the assumption that a major part of the peptide coils of the protein moiety are available to the surface of the molecule. The dialysis equilibrium experiments with methyl orange amply confirm this, although conclusions regarding the absolute binding capacity cannot be drawn, since the chlorine ion would tend to decrease the binding power, as has been shown for bovine albumin.[15] Yet, the decrease in binding capacity, as compared with bovine albumin, when a larger anion (Biebrich scarlet) is used would indicate that the cerebroside moiety of the lipoprotein does offer some spatial hindrance to the juxtaposition of the large organic anion with the cationic groups of the protein coil. It might also be argued that the fixation points on the surface of the lipoprotein molecule are sufficiently close so that both of the acidic groups of a single Biebrich scarlet molecule are attached, thus actually resulting in a smaller number of dye molecules being bound than would be expected from the number of acid-binding radicals on the lipoprotein molecule. However, one prefers to ascribe the phenomenon to the spatial hindrance offered by the large inert lipid moiety of the molecule, especially if the resistance shown by the Gaucher lipoprotein to the action of proteolytic enzymes is taken into account. The resistance of the lipoprotein to hydrolysis by various proteolytic enzymes, while the denatured protein moiety

15. Klotz, I. M.: Amino Acids and Proteins, in Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, L. I., New York, The Biological Laboratory, 1950, vol. 14, pp. 97-111. Uzman, L. L., and Cook, M.: Unpublished data.

offers no such resistance, can readily be ascribed to the protective effect of the lipid moiety in offering steric hindrance to the proper apposition of enzyme and substrate. In the past, considerations of the possible chemical pathogenesis of Gaucher's disease have invoked the concept of a defect in lipid metabolism, specifically a defect or deficiency of cellular cerebrosidases.[16] Although this view cannot be definitely rejected at this time, it is evident from the study of the isolated lipoprotein that the mutual steric protection offered by the lipid and protein moieties and the tenacity of the bond between them would be adequate to explain the continued presence of kerasin in the Gaucher cell, even though it were possible to demonstrate adequate intracellular enzyme levels and patterns. As yet, there is no explanation for the formation of the Gaucher lipoprotein, but once it has been formed, indications are that it presents an entity that would probably be resistant to the normal catabolic processes of the parent reticulum cell. Since kerasin has been shown to be a normal, if very small, constituent of splenic tissue,[17] and probably of other organs as well, the question arises whether Gaucher's disease may not be the manifestation of an inherent genetic defect of the protein-synthesizing matrix of the cells of the reticuloendothelial system resulting in the synthesis of a protoplasmic protein which has an abnormal affinity for kerasin and which in combining with this gives rise to the entity that has been described here.

The hexose present in the cerebroside moiety of the lipoprotein fractions isolated in both the present cases was galactose. Although an evaluation of the significance of the glucocerebrosides in Gaucher's disease is not germane to the present problem, it may be pointed out that the question does not appear to warrant the original controversy that arose between Klenk and Rennkamp [2e] and Lieb.[14] Since then, Klenk and Rennkamp [2f] have shown the glucocerebroside as well as the galactocerebroside forms of kerasin to be present in normal spleen, while Thannhauser and his associates,[18] in the case of twins with Gaucher's disease, found accumulation of only galactocerebrosides in the organs of one, while the other twin had both galactocerebrosides and glucocerebrosides in the various organs studied.

In any case, if one envisages the kerasin in the Gaucher cell to be firmly embedded in a mesh of peptide coils, as the present study seems to indicate, considerations of a defect in specifically active glucocerebrosidase or galactocerebrosidase systems lose much of their significance as possible explanations for the pathogenesis of Gaucher's disease.

SUMMARY

A lipoprotein fraction containing 62 per cent kerasin has been isolated from two spleens removed surgically from patients with Gaucher's disease. The cerebroside content of this fraction was found to account for more than 70 per cent of the kerasin present in each of the two spleens. Studies on the isolated lipoprotein

16. (*a*) Thannhauser, S. J., and Schmidt, G.: Physiol. Rev. **26**:275, 1946. (*b*) Ottenstein, B.; Schmidt, G., and Thannhauser, S. J.: Blood **3**:1250, 1948. (*c*) Thannhauser, S. J.: Lipidoses: Diseases of the Cellular Lipid Metabolism, in Christian, H. A.: Oxford Medicine, New York, Oxford University Press, 1949, vol. 4, pt. 2, pp. 214(3)-214(595). (*d*) Thannhauser, S. J., and Reichel, M.: J. Biol. Chem. **113**:311, 1936.

17. Klenk and Rennkamp.[2e, f]

18. Thannhauser and Schmidt.[16a] Ottenstein, Schmidt and Thannhauser.[16b]

fractions have shown a remarkable tenacity of the lipid-protein bond, which is broken only by prolonged boiling with chloroform-menthanol or by treatment with a protein denaturant such as eight times molar guanidine-hydrochloride. The native lipoprotein was found to be resistant to the action of proteolytic enzymes, although the organic anion-binding capacity of the fraction has indicated that a large part of the peptide coils are available at the surface of the lipoprotein macromolecule. It is concluded that the cerebroside which chemically characterizes the Gaucher cell is firmly bound within the cell in the form of a lipoprotein, the rather distinctive properties of which will have to be considered in any future formulation of concepts regarding the pathogenesis of Gaucher's disease.

In most of the analytical work the author had the assistance of Miss Marilyn Cook.

EPITHELIAL METAPLASIA IN "PROSTATIC INFARCTION"

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SQUAMOUS cell carcinoma of the prostate is an extremely rare lesion. Nevertheless, this diagnosis is frequently made because squamous metaplasia occurring in prostatic infarction simulates squamous cell carcinoma.[1]

Fifty cases of prostatic infarction in which specimens have been seen at the Armed Forces Institute of Pathology during the last four years furnished the basis for this investigation. Epithelial metaplasia was an outstanding feature and was observed in 47 of the prostates, whereas it was absent in three in which the infarction was septic. In eight cases in which metaplasia was noted, there was a coexisting adenocarcinoma of the prostate; in 10 others a diagnosis of carcinoma had been made erroneously by one or more examiners.

This discussion will be limited to the 47 cases in which epithelial metaplasia was associated with prostatic infarction. The clinical aspects may be summarized briefly. Prostatism was present in every patient in this series, the youngest of whom was 37 and the oldest 80. The most important single symptom was transient acute urinary retention, which was noted in 50 per cent of the patients. Almost half of these patients gave a history of operative instrumentation, such as transurethral resection, catheterization or cystoscopy.

Morphologically, the lesion was always situated in a hyperplastic nodule, but in the available tissue it was impossible to determine the relative frequency with which it occurred in various lobes. The lesion was spherical, measured as much as 2.5 cm. in diameter and was centrally placed. In some instances it was hemorrhagic; in others it was pale gray mottled with dark red, or yellowish. It was usually single, but in five instances from two to four distinct lesions were found.

From the Armed Forces Institute of Pathology and the Central Laboratory, Veterans Administration.

Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or the policy of the Veterans Administration.

1. Abeshouse, B. S.: Infarct of the Prostate, J. Urol. **30**:97-112, 1933. Culp, O. S.: Squamous Metaplasia Simulating Carcinoma, Associated with Prostatic Infarction, Bull. Johns Hopkins Hosp. **65**:239-252, 1939. Moore, R. A.: Benign Hypertrophy of Prostate: Morphological Study, J. Urol **50**:680-710, 1943. Roth, R. B.: Prostatic Infarction, ibid. **62**:474-479, 1949. Hubly, J. W., and Thompson, G. J.: Infarction of the Prostate and Volumetric Changes Produced by the Lesion: Report of 3 Cases, ibid. **43**:459-467, 1940.

The histological sequence of development of the lesion is demonstrated in figures 1 through 4. In the early phase there is an area of more or less complete coagulation or ischemic necrosis involving both the fibromuscular stroma and the epithelial tissue. Marginal or diffuse hemorrhage may or may not be a feature. Occasionally polymorphonuclear granulocytes infiltrate the area, but the infiltration is rarely intense. The acini at the periphery display vacuolation, fragmentation, compression and distortion (fig. 1).

During the stage of healing, hyperplasia and metaplasia of the epithelium can be observed in many of the marginal acini (fig. 2). As healing progresses the

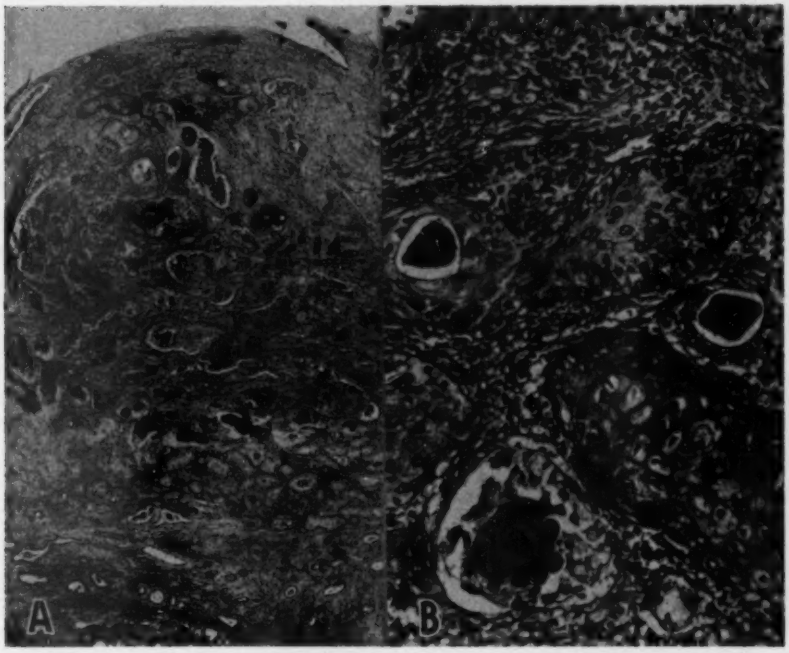

Fig. 1.—*A*, central area of coagulation necrosis in the prostate of an 80 year old man who had no history of previous operative intervention. AFIP accession 189106; × 21.

B, same section, higher power. Squamous cells fill the acinar spaces. Structures suggesting intercellular bridges are apparent. AFIP accession 189106; ×180.

necrotic area is replaced by fibrous tissue containing solid or acinar nests of epithelial cells (fig. 3*A*).

Metaplastic epithelium of three types is present in the lesion. The first, found in the majority of cases, consists of cells that closely resemble squamous cells, arranged in from three to six layers. The cells are polygonal with acidophilic or vacuolated cytoplasm and large vesicular nuclei. Intercellular bridges and even pearl formation may be present. Some of these cells appear to be free in the lumen. Epithelium of the second type is made up of cells which are elongated

and arranged in whorls and which resemble transitional cells. That of the third type consists of low columnar cells which line acinar structures, resembling the formations found in Brunner's nests.

The cells display no anaplasia, hyperchromatism or increased or abnormal mitotic activity, and they do not invade the surrounding stroma. The fibromuscular stroma of the prostate in these areas is completely replaced by fibrous connective tissue, as demonstrated by Masson stains. In some areas the stroma is cellular; elsewhere it is hyalinized. There may be recent hemorrhage or hemosiderin deposits within or outside macrophages. A few lymphocytes may be present.

Fig. 2.—*A*, recent, probably operative, hemorrhage in the center of an area of scarring in the prostate of a 77 year old man. Transurethral resection had been performed six years earlier, and transient retention developed four months before this operation. AFIP accession 188738; × 5.

B, an area of *A* under higher power. Cuboidal cells line acinar spaces, sometimes filling them. AFIP accession 188736; × 115.

COMMENT

The metaplasia of the marginal acini and ducts seen in "prostatic infarction" is situated around areas of coagulation necrosis or fibrous replacement of the fibromuscular stroma. Characteristically, it occurs in a hyperplastic nodule and is associated with necrosis or fibrous tissue scar. The cells show no anaplasia, no hyperchromatism and no increased or abnormal mitotic activity, and they do not break through the basement membrane. In carcinoma there is hyperchromatism,

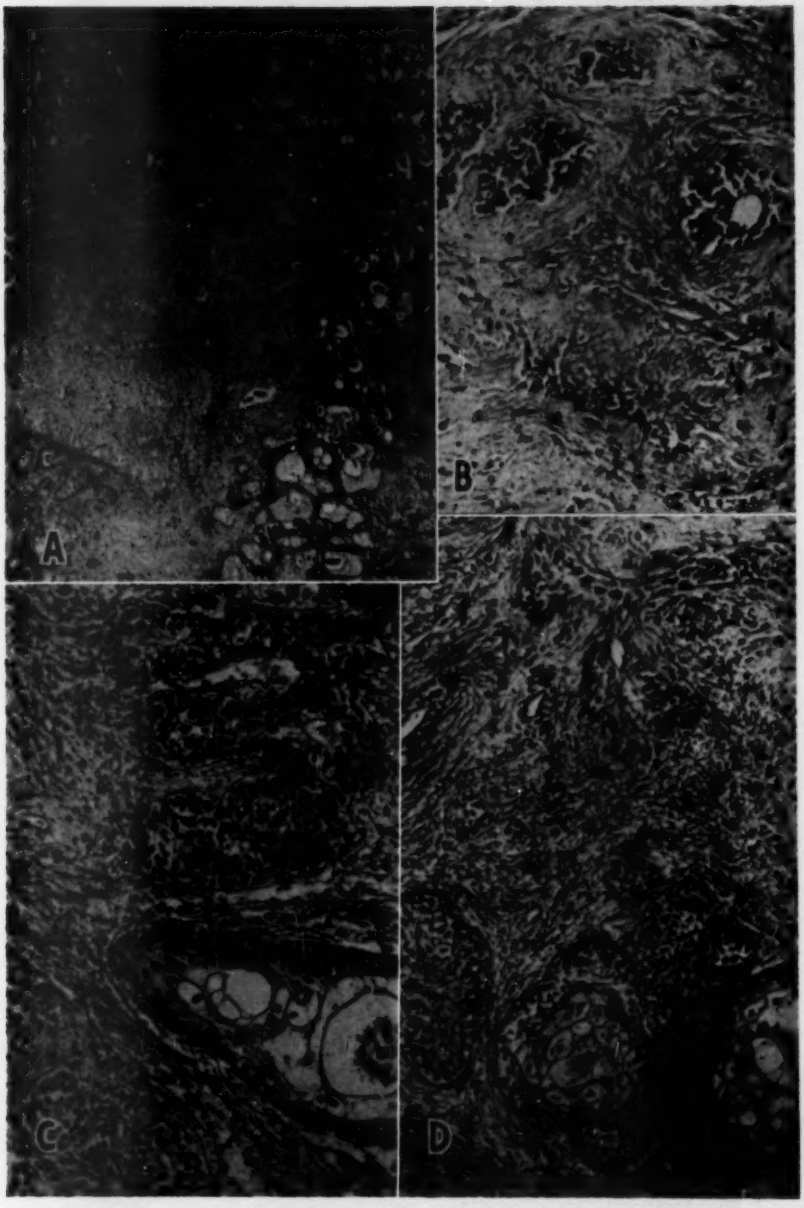

Fig. 3.—*A*, complete fibrous replacement of the central part of a nodule of the prostate. AFIP accession 283264; × 21.

B, hyalinized stroma and acini resembling Brunner's nests. AFIP accession 283264; × 115.

C and *D*, squamous cells and transitional cells replacing the normal acinar epithelium. The stroma is quite cellular. AFIP accession 283264; × 115.

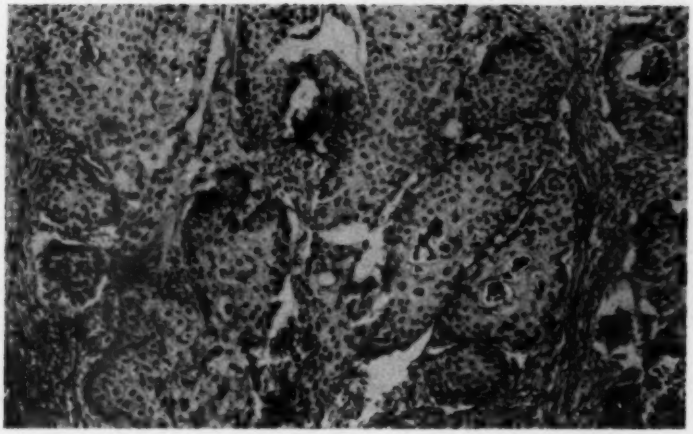

Fig. 4.—Islands of epithelial cells are surrounded by cellular stroma. Although the stroma suggests smooth muscle, a Masson stain shows absence of any muscle elements. AFIP accession 283138; × 115.

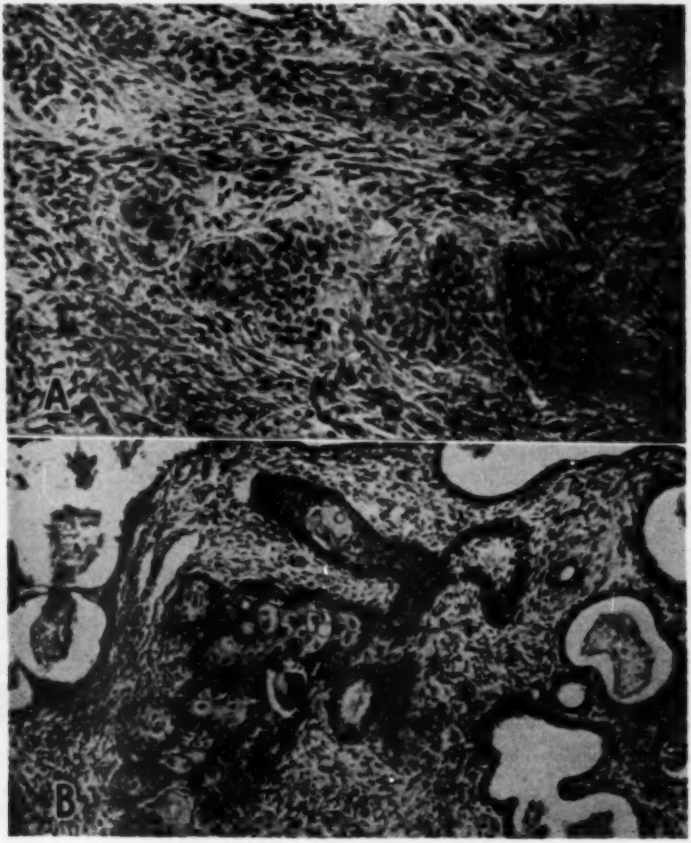

Fig. 5.—*A*, nests of transitional cells without any apparent lumen are surrounded by a cellular fibrous tissue stroma. AFIP accession 176373; × 115.
B, area of squamous metaplasia. AFIP accession 124502; × 115.

loss of polarity, variation in size and shape of cells, increased and abnormal mitotic activity and invasion of the fibromuscular stroma.

The epithelial changes occurring in prostatic infarction are not of a cancerous nature, but the finding of an area of infarction does not and should not rule out the presence of carcinoma elsewhere in the prostate. In eight patients of this group an unmistakable adenocarcinoma of the prostate coexisted with infarction and squamous metaplasia. The lesion should also be distinguished from the occasional prostate-invading carcinoma of the bladder or of the posterior urethra. Another condition to be differentiated is the squamous metaplasia associated with diethylstilbestrol therapy, but this is a diffuse process, involving, however, only part of each acinus, while squamous metaplasia occurring spontaneously (i. e., unassociated with infarction of the prostate) is usually limited to the ducts. Both may be accompanied by squamous metaplasia of the urethra, and there is no necrosis or fibrous replacement of the stroma.

The mechanism of the squamous metaplasia and the length of time that intervenes before this change becomes apparent in infarction of the human prostate remain obscure. In two of our patients metaplasia was apparent at prostatectomy when instrumentation was known to have been performed three days previously in one instance and four in the other.

Despite the fact that such lesions have been called infarcts, no thrombosis or occlusion of the arteries has ever been demonstrated. It has been presumed, however, that in cases in which operative intervention preceded the appearance of the lesion, loss of blood supply as a result of operative sectioning or coagulation of penetrating prostatic arteries could account for the formation of the infarct. When there is no history of previous instrumentation, it is possible that the unyielding capsule of a hyperplastic nodule might interfere with the blood supply and thus account for infarction. Our material did not lend itself to a thorough examination of the vascular supply of the prostate. Where facilities are available, investigation of this problem is highly desirable, so as to determine whether there is any justification for the use of the term infarct.

SUMMARY

Squamous metaplasia of prostatic epithelium occurs in the so-called infarction of the prostate and is associated with an area of necrosis or scar. This lesion is to be distinguished from squamous cell carcinoma.

THE TOXICOLOGY OF 1,2-DICHLOROETHANE (ETHYLENE DICHLORIDE)

V. Effect of Protective Agents on Visceral Fatty Changes in Exposed Rats

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ETHYLENE dichloride (1,2-dichloroethane [$CH_2Cl.CH_2Cl$]) is a colorless liquid somewhat resembling chloroform in odor and narcotic action. It is widely used as a solvent and for various industrial purposes, and hence its toxic properties are of interest. In laboratory animals it produces fatty degeneration of the liver, the kidney and the heart and, less commonly, necrosis of the inner cortical tubules of the kidney, pleural effusion and, in dogs and foxes, corneal opacity of the eyes.[1] In previous reports from this laboratory, Heppel and associates [2] showed that among mice and young rats inhaling dichloroethane for one or more periods daily the mortality rate was reduced by dl-methionine, by certain sulfhydryl compounds like l-cystine and by several amino-substituted benzene derivatives like aniline and sulfanilamide. This paper is concerned with a study of the effects of such protective agents on the histological changes induced by dichloroethane.

METHODS

The technic evolved is somewhat similar to that used by Brunschwig, Johnson and Nichols [3] in their study of the action of certain agents protecting against carbon tetrachloride injury of the liver. In each experiment, 20 to 50 Sprague-Dawley male rats weighing 60 to 120 Gm. were selected and divided into groups of four to 15 rats each. One group serving as a control was given a subcutaneous injection of 1 cc. of dichloroethane per kilogram of rat weight and usually isotonic sodium chloride solution or 5 per cent acacia in isotonic sodium chloride solution to match the other experimental groups. Each of the other groups received one of the protective agents immediately after the injection of the dichloroethane. Whenever applicable, the dose was similar to that used by Heppel and his associates. Most of the agents were given by stomach tube in aqueous solution or suspended in 5 per cent acacia in isotonic sodium chloride solution, the volume not exceeding 5 cc. for a 100 Gm. rat at one time. If the total dose exceeded 1,000 mg. per kilogram, the excess was generally given four to six hours later. The sodium bicarbonate solution was given in two equally divided doses at four hour intervals.

From the Experimental Biology and Medicine Institute, National Institutes of Health.

1. (*a*) Heppel, L. A.; Neal, P. A.; Endicott, K. M., and Porterfield, V. T.: Toxicology of Dichloroethane: Effect on the Cornea, Arch. Ophth. **32**:391 (Nov.) 1944. (*b*) Heppel, L. A.; Neal, P. A.; Daft, F. S.; Endicott, K. M.; Orr, M. L., and Porterfield, V. T.: J. Indust. Hyg. & Toxicol. **27**:15, 1945. (*c*) Heppel, L. A.; Neal, P. A.; Perrin, T. L.; Endicott, K. M., and Porterfield, V. T.: J. Pharmacol. & Exper. Therap. **84**:53, 1945.

2. Heppel, L. A.; Porterfield, V. T., and Sharpless, N. E.: J. Pharmacol. & Exper. Therap. **91**:385, 1947. Heppel and others.[1c]

3. Brunschwig, A.; Johnson, C., and Nichols, S.: Proc. Soc. Exper. Biol. & Med. **60**:388, 1945.

Surviving rats were killed 24 hours after the injection of the dichloroethane. Hearts, livers and kidneys were fixed in a solution of formaldehyde, and frozen sections of these organs were graded for fat (0 to 4 +) after staining with oil red O.[4]

RESULTS

The control rats given 1 cc. of dichloroethane per kilogram had an average mortality rate of about 35 per cent in 24 hours, a high incidence of fatty changes in heart, liver and kidney and a relatively low incidence of renal necrosis. Fatty changes were minimal or absent in rats killed 48 hours after the injection. Larger doses of dichloroethane caused a much higher mortality rate, and smaller doses

TABLE 1.—*Effect of Various Agents Used to Protect Against Dichloroethane* *

Agent †	Dose, Mg. per Kg.	Mortality Rate in 24 Hr.	Survivors Studied	Percentage of Survivors with Moderate to Severe Fatty Changes in			Percentage of Survivors with Renal Necrosis
				Heart	Liver	Kidney	
Control	.....	24	29	41	46	49	13
dl-Methionine	1,500	5 ‡	39	0	15	38	48 ‡
Control	.....	45	23	52	61	61	9
dl-Methionine	1,500	30	31	0	10	6	29
Control	.....	52	16	81	81	81	6
1,2-Dimercaptopropanol	100	29 ‡	34	15	29	26	0
Control	.....	40	35	50	51	54	14
l-Cysteine.HCl	2,000	24 ‡	47	0	26	36	15
Control	.....	53	28	39	46	54	7
l-Cystine	2,000	16	37	11	41	41	27 ‡
Control	.....	38	34	39	47	53	18
Aniline	500	17	45	9	13	40	20
Control	.....	34	29	45	52	59	7
Sulfanilamide	1,000	24	32	22	25	34	22
Control	.....	9	20	50	45	50	15
Paraaminobenzoic acid	2,000	6	33	12	30	36	3
Control	.....	22	49	45	51	49	35
Sodium bicarbonate	3,700	25	40	39	33	33	22
Control	.....	29	14	50	36	43	14
Choline chloride	500	35	15	40	33	43	43
Control	.....	58	18	61	50	56	17
Sodium thiosulfate	200	41	22	32	45	57	26

* Surviving rats were killed for study 24 hours after a subcutaneous injection of 1 cc. of dichloroethane per kilogram of body weight.

† All the agents were given by stomach tube except dl-methionine, which was given intraperitoneally in a 2 per cent aqueous solution to 41 rats, and 1,2-dimercaptopropanol (BAL), which was given intramuscularly in two divided doses at four hour intervals, dissolved in 10 per cent benzyl benzoate in peanut oil. Sulfanilamide and l-cystine were suspended in 5 per cent acacia in isotonic sodium chloride solution, and paraaminobenzoic acid was dissolved in a 2.7 per cent aqueous solution of sodium bicarbonate. All the other agents were dissolved in water.

‡ Statistically significant. The significance of the fatty changes is shown in table 2.

produced a much lower incidence of fatty changes. The fatty changes were usually patchy in the heart and irregular or diffuse in the liver lobules. In the kidney, nearly all the tubules in the outer portion of the renal cortex were heavily laden with fine droplets of fat in severe cases. Renal necrosis when present involved the inner cortical tubules and was usually associated with some fatty changes in the adjacent medulla, confined largely to portions of the loops of Henle. In some cases of renal necrosis, there was little or no fat in the outer cortical tubules.

The data in table 1 were obtained from 26 experiments in which rats received subcutaneously 1 cc. of dichloroethane per kilogram. The figures for each compound include data from three to nine separate experiments in which the particular substance was tested; on the line above each compound listed are the corresponding

4. Lillie, R. D.: Histopathologic Technic, Philadelphia, The Blakiston Company, 1948.

data for animals used as controls in the same experiments. The organs of the rats that died after 12 hours generally showed severe fatty changes but are not included in this study.

As indicated by statistical analysis (table 2), the compounds most effective in inhibiting fatty changes were methionine, dimercaprol (BAL), cysteine and aniline. Aniline and dimercaprol were usually given by stomach tube and intramuscularly, respectively; they were similarly effective when given intraperitoneally, but the mortality rate tended to be higher. The reductions in mortality rate (table 1) were significant statistically (could have occurred by chance less than one time in 20) in the rats receiving methionine (intraperitoneally), cysteine and dimercaprol.

Many other compounds were investigated. Preliminary tests indicated that a few of these compounds, such as glutathione (500 to 1,000 mg. per kilogram), may

TABLE 2.—*Statistical Analysis of Data on Fatty Changes in Heart, Liver and Kidney Shown in Table 1*

		Heart		Liver		Kidney	
Agent	Experiments	Av. Difference,* %	Stand. Error of Difference, %	Av. Difference,* %	Stand. Error of Difference, %	Av. Difference,* %	Stand. Error of Difference, %
dl-Methionine †	9	40 ‡	10.8	28 ‡	11.1	12	17.9
dl-Methionine	6	64 ‡	11.5	60 ‡	8.2	68 ‡	7.2
1,2-Dimercaptopropanol	4	63 ‡	11.8	52 ‡	7.9	54 ‡	8.3
l-Cysteine.HCl	7	53 ‡	10.8	30 ‡	5.9	19 §	9.6
l-Cystine	6	34 §	10.8	7	14.5	18	14.6
Aniline	6	40 §	14.2	44 ‡	11.2	12	12.1
Sulfanilamide	5	19 §	5.7	18	19.0	18	16.3
Paraaminobenzoic acid	4	41 §	12.0	10	8.6	17	8.7
Sodium bicarbonate	6	13	10.3	18 §	6.7	17	8.4
Choline chloride	3	15	10.3	3	12.4	—2	....
Sodium thiosulfate	4	9	11.3	—2	....	—4	....

* The average difference in percentage was computed by weighting the difference in incidence of fatty changes for each experiment by the harmonic mean of the number of treated and control animals in each experiment.

† This was injected intraperitoneally.

‡ Highly significant statistically. Any material which showed a degree of protection which could have occurred by chance alone less than one time in 100, as demonstrated by a one-sided test of significance, was considered as highly significant.

§ Significant. An agent which showed a decrease in incidence of fatty changes which could have occurred by chance less than five times but more than one time in 100 was considered significant.

Any agent which showed a degree of protection which could have occurred by chance more than five times in 100 is considered as not significant (not marked in the table).

lower the incidence of fatty changes, particularly in the heart, but none of the compounds appeared to be as effective as methionine. Most of the other compounds appeared to be ineffective or were too toxic under the experimental conditions to be given in substantial doses. For example, sodium thioglycollate (150 mg. per kilogram) and 2-thiobarbituric acid (300 mg. per kilogram) were not effective; larger doses greatly increased the mortality rate from dichloroethane. Even small doses of thiourea and thiouracil (50 mg. per kilogram) proved highly lethal when the initial dose was given with the dichloroethane. In one experiment, 500, 250 and 50 mg. of thiourea per kilogram were given to eight rats by stomach tube 48, 24 and 0 hours before the injection of the dichloroethane; none of the eight rats died, and severe fatty changes were less frequent than in the corresponding controls.

Renal necrosis occurred in rats receiving sodium thioglycollate and vitamin B_{12}, which have been reported to be effective in protecting the liver against injury

by chloroform [5] and carbon tetrachloride.[6] The incidence of renal necrosis was significantly increased in rats receiving methionine or cystine.

COMMENT

Our findings indicate that the mortality rate and the visceral incidence of fatty changes in young male rats 24 hours after a subcutaneous injection of dichloroethane may be signficantly reduced by methionine and certain sulfhydryl compounds like cysteine. Heppel, Porterfield and Sharpless [2] noted that in young rats these compounds lowered the mortality rate due to inhalation of dichloroethane but that small doses of 1,2-dimercaptopropanol (BAL) (1 mg. per rat) did not protect. We found similar doses of the latter valueless, but larger amounts (10 mg. per 100 Gm. rat) were most effective. Heppel and his associates found 2-thiobarbituric acid, thiourea and thiouracil protective when administered in the diet beginning three days before the onset of dichloroethane inhalation exposure. Under the conditions of our experiment, 2-thiobarbituric acid was ineffective in the dose tolerated, and even small doses of thiourea and thiouracil greatly increased the mortality rate from dichloroethane. Thiourea was better tolerated, however, when its administration was begun 48 hours before the dichloroethane. Heppel and his associates found that aniline, sulfanilamide and paraaminobenzoic acid protected mice, but that paraaminobenzoic acid did not protect rats against inhaled dichloroethane. Our findings indicate that the three compounds are of value in lowering the incidence of fatty changes, particularly in the heart, although the reduction in mortality rate is not statistically significant on the basis of the limited data available. Sodium bicarbonate was used to dissolve the paraaminobenzoic acid; it was noted that controls receiving sodium bicarbonate showed a lower incidence of fatty changes in the liver than the controls receiving water. Sodium bicarbonate has not been tested previously as an agent protecting against dichloroethane. Our findings concerning the ineffectiveness of sodium thiosulfate and choline agree with previous studies.[2]

All the tested agents causing a significant reduction in mortality rate also produced a more consistent and pronounced reduction in the incidence of fatty changes in the heart. The failure to reduce the incidence of fatty changes in the heart repeatedly proved a reliable early indication that the compound being tested would prove ineffective in lowering the mortality rate due to dichloroethane. It must be emphasized, however, that the mortality rate was not lowered by all the compounds that decreased the incidence of fatty changes in the heart.

The occurrence of renal necrosis in some kidneys showing little or no fatty changes and the failure of certain protective agents like methionine to prevent renal necrosis suggest that the factors causing renal necrosis differ from those causing fatty changes.

SUMMARY

In young white male rats a subcutaneous injection of 1 cc. of 1,2-dichloroethane per kilogram of body weight produces in 24 hours a mortality rate of about 35

5. Brunschwig, A.; Nichols, S.; Bigelow, R. R., and Miles, J.: Sulfhydryl Protection of the Liver, Arch. Path. **40**:81 (Aug.) 1945.

6. Popper, H.; Koch-Weser, D., and Szanto, P. B.: Proc. Soc. Exper. Biol. & Med. **71**:688, 1949. Brunschwig and others.[5]

per cent and a high incidence of severe fatty degeneration of the heart, the liver and the kidney. The mortality rate and the incidence of fatty changes were reduced by administering, immediately after the injection of dichloroethane, methionine and certain sulfhydryl compounds like dimercaprol (BAL) and cysteine. The incidence of fatty changes in the heart or the liver was reduced signficantly by aniline, sulfanilamide, paraaminobenzoic acid and sodium bicarbonate. The incidence of renal necrosis due to dichloroethane was increased by some agents, such as methionine. Some compounds, like thiourea, greatly increased the mortality rate when first given immediately after the dichloroethane. Thiourea was better tolerated when the initial dose was given 48 hours before the dichloroethane. Nonprotective compounds could often be screened out quickly by their failure to lower appreciably the incidence of fatty changes in the heart.

The analysis of data given in table 2 was prepared by Jerome Cornfield, chief, Research Studies Unit, Biometrics Section, National Cancer Institute.

QUANTITATIVE EXFOLIATIVE CYTOLOGY

An Evaluation of the Glycogen Content of Exfoliated Epithelial Cells in Urine Sediment from Pregnant and Nonpregnant Women

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THIS communication records an evaluation of the glycogen content of exfoliated epithelial cells in urine sediment as a possible diagnostic aid in pregnancy. Cyclical changes in morphologic aspect that can be correlated with the menstrual cycle have been reported in smears of the sediment of urine from nonpregnant women.[1] Diagnosis of pregnancy by specific cytologic criteria in examination of urinary sediment has been reported by Papanicolaou,[2] who described the presence of navicular cells closely analogous to certain cells seen in smears of vaginal material from pregnant women. Other investigators [1] recorded similar morphologic changes in urinary cells in pregnancy, especially in the later stages of gestation. These reports seem to indicate that the cells in urinary sediment tend to reflect the morphological changes of cells in vaginal smears in pregnancy and in the menstrual cycle.

It has been known for some time that glycogen is increased in certain cells of the female genital tract in pregnancy.[2] From the foregoing, it is logical to suppose that exfoliated bladder cells from pregnant women might reflect the specific cytochemical change of increased glycogen deposition. In this connection Papanicolaou [2a] has reported that during gestation there is evidence of an increased production of glycogen in the epithelium of the bladder, parallel to that in the epithelium of the cervix and vagina.

A technic for computing the percentage of exfoliated cells containing glycogen has been reported by Foraker and Brawner.[3] We found a statistically significant increase in glycogen-containing cells from the cervix of the pregnant as compared with the nonpregnant woman. The present study applies similar methods to exfoliated epithelial cells in sediment of urine from pregnant and nonpregnant women.

MATERIALS AND METHODS

Urine specimens were obtained by catheterization from 30 pregnant women (ranging from the third to the ninth month of gestation) and from 24 nonpregnant women of similar age

From the Departments of Pathology, Emory University School of Medicine and Grady Memorial Hospital.

1. McCallin, P. F.; Taylor, E. S., and Whitehead, R. W.: Am. J. Obst. & Gynec. **60**:64-74, 1950.

2. (*a*) Papanicolaou, G. N.: Proc. Soc. Exper. Biol. & Med. **67**:247-249, 1948. (*b*) Wislocki, G. B.; Bunting, H., and Dempsey, E. W., in Engle, E. T.: Menstruation and Its Disorders, Springfield, Charles C Thomas, Publisher, 1950, pp. 23-44.

3. Foraker, A. G., and Brawner, D. L.: Arch. Path., to be published.

group. The specimens were immediately centrifuged and sediment smears prepared and fixed in alcohol-ether by a standard cytologic technic.[4] In each case half of the slides were digested with saliva before staining as a control procedure, because certain substances give false positive reactions with glycogen stains.[5] All slides were stained by a modification of the periodic acid routine,[6] which is an accepted method of demonstrating glycogen in tissue.[6]

All counts were performed by the same person (A. G. F.), who did not know the patients' clinical status. No attempt was made to identify specific epithelial cell type or to record cytologic detail. In all smears the cells staining positively (purple) were readily differentiated from those failing to take the stain. The number of borderline cells not clearly taking or failing to take the stain was negligible. In all but a few cases, more than 2,000 cells were counted in the material from each patient. On the average 2,196 cells were counted in each case. A total of 118,579 exfoliated epithelial cells were counted in this study.

RESULTS

The findings are summarized in the table. In urine sediment smears representing 30 pregnant women a mean of 51.38 ± 13.79 per cent cells staining positively

Mean Percentage of Epithelial Cells in Urinary Sediment from 30 Pregnant and 24 Nonpregnant Women Staining Positively with the Periodic Acid Routine

	Before Saliva Digestion	After Saliva Digestion	"Glycogen-Containing" Cells
Total nonpregnant women (24)	51.42 ± 12.52	3.77 ± 3.07	47.65 ± 13.09
Total pregnant women (30)	51.38 ± 13.79	3.58 ± 3.83	47.80 ± 14.78
3d month gestation (2)	44.14 ± 8.14	4.21 ± 2.44	39.98 ± 5.65
4th month gestation (3)	62.86 ± 3.26	2.39 ± 1.02	60.47 ± 4.96
5th month gestation (5)	53.15 ± 9.88	6.21 ± 5.64	46.94 ± 9.51
6th month gestation (5)	44.50 ± 14.15	2.29 ± 1.88	42.21 ± 16.36
7th month gestation (7)	53.73 ± 16.50	4.21 ± 5.79	49.52 ± 15.73
8th month gestation (5)	55.00 ± 18.24	1.77 ± 1.82	53.23 ± 18.79
9th month gestation (3)	41.72 ± 9.61	3.66 ± 2.26	38.06 ± 10.79

with periodic acid was found. Smears representing 24 nonpregnant women contained a mean of 51.42 ± 12.52 per cent positively staining cells. This figure represents the percentage of cells containing glycogen in addition to other substances staining with the periodic acid routine. Following saliva digestion the smears representing the pregnant women contained a mean of 3.58 ± 3.83 per cent positively staining cells. The saliva-digested smears representing the nonpregnant women showed a mean of 3.77 ± 3.07 per cent positively staining cells. This figure represents the percentage of cells containing substances other than glycogen which react positively to the periodic acid routine. The difference between the percentages of positively staining cells in the undigested and digested smears represents the percentage of those cells owing their staining properties to glycogen alone. Using this computation, the smears representing the pregnant women contained 47.80 ± 14.78 per cent "glycogen-containing" cells. The smears representing the nonpregnant women revealed 47.65 ± 13.09 per cent "glycogen-containing"

4. Graham, R. M., and others: The Cytologic Diagnosis of Cancer, Philadelphia, W. B. Saunders Company, 1950, p. 207.

5. Lillie, R. D.: Histopathologic Technique, Philadelphia, The Blakiston Company, 1948, p. 145.

6. McManus, J. F. A., and Findley, L.: Surg., Gynec. & Obst. **89**:616-620, 1949.

cells. Our results indicate no significant difference in the number of glycogen-containing cells in the sediment of urine from these pregnant and nonpregnant women.

COMMENT

Since smears of sediment of urine from pregnant and nonpregnant women showed little difference in percentages of cells staining positively with periodic acid, both before and after saliva digestion, it cannot be expected that further computations involving these factors will show good differentiation.

The number of cases representing each month of gestation is too small to justify an analysis of the means recorded by month. However, there is no evidence of a trend toward increased glycogen deposition in bladder cells as pregnancy progresses. Marked variation was found in the percentages of positively staining cells before and after saliva digestion in individual cases. Considering this variation, the degree of conformity of the means for the pregnant and nonpregnant women is striking ($t = .007598$, $p < .001$).[7]

The marked variations in staining reaction in individual cases, both those of pregnant and those of nonpregnant women, may hold some meaning. These differences could result from individual variations in carbohydrate metabolism. It has previously been reported that excess of ingested carbohydrate had no effect on the glycogen content of vaginal epithelium in women.[8] It is possible that the glycogen content of the bladder epithelium of an individual may vary significantly from time to time. It is also possible that the glycogen content of an individual woman's exfoliating bladder epithelium may change during gestation.

In this series, exfoliated epithelial cells in urinary sediment did not show evidence of increased glycogen content in pregnancy. Cells in urinary sediment have been said to be almost completely of bladder origin.[1] We have not located the exact origin of the cells counted in this study, but we assume that a majority of them derive from the bladder. Unless one postulates an increased proportion of non-glycogen-containing cells from the upper urinary tract during pregnancy, this study did not show evidence of increased glycogen deposition in bladder epithelium during gestation. Our results, therefore, have failed to confirm previously reported evidence[2a] that there is increased glycogen production in bladder epithelium during gestation.

SUMMARY

Differential counts of urine sediment smears stained for glycogen, representing 30 pregnant and 24 nonpregnant women, showed almost exactly equal mean percentages of glycogen-containing cells. The results of this study reveal no increase of glycogen deposition in the epithelial cells of urinary sediment during gestation.

7. Snedecor, G. W.: Statistical Methods Applied to Experiments in Agriculture and Biology, ed. 4, Ames, Iowa, The Iowa State College Press, 1948, p. 65.

8. Willson, J. R., and Goforth, M. L.: J. Clin. Endocrinol. **2**:223-225, 1942.

Notes and News

Deaths.—Francis Carter Wood, New York, internationally known authority on cancer and pioneer in the use of radium and roentgen rays, died January 5, aged 81, of coronary thrombosis. Dr. Wood was associated with St. Luke's Hospital, serving as director of the radiotherapeutic department since 1921 and of the pathological laboratory since 1910. He was professor of clinical pathology with the College of Physicians and Surgeons; from 1912 to 1940 he was director of the Columbia University Institute of Cancer Research, and from 1925 to 1932 Dr. Wood was a member of the Council on Physical Therapy of the American Medical Association. He was the editor of Delafield and Prudeen's "Textbook of Pathology," sixteenth edition, 1935, and of the *American Journal of Cancer* from 1930 to 1941. His latest work, "An Atlas on Tumor Pathology," will be ready for publication soon. Dr. Wood was a member of numerous national and international societies, including the American Association of Pathologists and Bacteriologists, the Society of Experimental Biology and Medicine, the New York Pathological Society, the American Cancer Society, the American Society for the Control of Cancer and the Association of American Physicians and was on the advisory committee to the New York State Division on Cancer Control.

Dr. John D. Allen, Louisville, Ky., died October 15 last, aged 66, of coronary occlusion. He was a specialist certified by the American Board of Pathology, a member of the College of American Pathologists, and past president of the Jefferson County Medical Society.

Dr. Ralph Griffiths Stillman died in Kent, Conn., November 17 last, aged 68, of carcinoma of the lungs. Dr. Stillman was assistant professor of medicine (clinical pathology) at Cornell University Medical College; a specialist certified by the American Board of Pathology; a member of the American Association of Pathologists and Bacteriologists and the American Society of Clinical Pathologists.

Dr. Leon Stanley Lippincott, Daytona Beach, Fla., a specialist certified by the American Board of Pathology, died Nov. 25, 1950, aged 62, of phlebitis and pulmonary embolism. Dr. Lippincott was formerly on the faculty of the Medical School of Maine, Portland, and Tufts College Medical School, Boston. He was a fellow of the American Society of Clinical Pathologists, the American College of Physicians and the American Public Health Association. He was a founding fellow of the College of American Pathologists.

Announcement of Meetings in Texas.—Several meetings of interest to scientists and physicians will be held at the Texas Medical Center, Houston, Texas, April 20 and 21:

The Fifth Annual Symposium on Fundamental Cancer Research of the University of Texas M. D. Anderson Hospital for Cancer Research will devote one-half day to selected papers on the subject of protein metabolism in cancer.

The Cancer Pathology Conference of the University of Texas Postgraduate School of Medicine will be concerned primarily with breast cancer. An assortment of other tumors will be discussed, however.

A South Central Region Meeting of the College of American Pathologists will be held, with co-participation in the symposium and the pathology conference, mentioned above. Papers of general interest to pathologists will be included in this program. Some of the featured speakers will be Dr. Paul C. Aebersold, Oak Ridge Institute of Nuclear Studies; Dr. J. J. Bittner, University of Minnesota Medical School; Dr. Frank W. Foote, Memorial Hospital for the Treatment of Cancer and Allied Diseases, New York; Dr. W. C. Hueper, National Cancer Institute; Dr. Fred W. Stewart, Memorial Hospital for the Treatment of Cancer and Allied Diseases, New York; Dr. Roger J. Williams, of the University of Texas.

Dr. Fred W. Stewart will deliver the second Bertner Foundation Lecture at the banquet on April 21. All meetings will be held at the Shamrock. Further information may be obtained from William O. Russell, M.D., 2310 Baldwin Street, Houston, Texas.

Books Received

MEDICAL GYNECOLOGY. By James C. Janney, M.D., associate professor of gynecology, Boston University School of Medicine; associate visiting gynecologist, Massachusetts Memorial Hospital. Second edition. Pp. 454 with 108 illustrations. Price, $6.50. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia 5; 7 Grape St., Shaftesbury Ave., London, W.C. 2, 1950.

Dr. Janney has approached his subject from the standpoint of office practice of gynecology and the problems which arise in the daily contacts with gynecologic patients. His style is simple and direct, and his analysis of the relative importance of various items entering into diagnosis and medical management shows wide experience and much thoughtful consideration of the subjects covered.

The book should be particularly useful to practitioners who do a considerable amount of office practice of gynecology. It is not meant to be an exhaustive treatise on the subject but rather to set a pattern for the study of patients who present themselves with common gynecologic complaints.

The sociomedical problems are well covered, especially the marriage problems.

A chapter devoted to various diagnostic tests gives an excellent idea of their usefulness in daily practice, and little details mentioned in the text indicate a firsthand knowledge of the subject.

One could wish that the illustrative material had been more carefully selected to supplement the written text. Many of the pictures are from old books and were good in their original setting, but a few original illustrations would have improved the appearance of the book.

One might disagree with a few statements, such as that urethrocele is a prolapse of the urethral mucosa and that varicose veins are a vulval tumor.

This book can be recommended as valuable for collateral reading in medical school classes or as a ready reference for the young physician starting practice.

BACTERIAL POLYSACCHARIDES: THEIR CHEMICAL AND IMMUNOLOGICAL ASPECTS. By Martin Burger, formerly organic chemist to the Bureau of Laboratories, New York. Pp. 273, with 48 tables. Price $6. Charles C Thomas, 301-327 E. Lawrence Ave., Springfield, Ill., 1950.

This book is divided into 15 chapters reviewing the literature of the serologically active polysaccharides obtained from bacteria of medical importance. It also contains a valuable appendix which gives the most successful methods of preparing the best known polysaccharides from pathogenic bacteria. Each chapter covers roughly the historical background of the organisms in question, the literature on the methods of isolating the serologically active polysaccharides from the bacteria discussed, and finally a more or less detailed presentation of the chemical and serologic characteristics of the isolated carbohydrates. The book is well documented, with many references in each chapter.

By bringing together a large amount of published information on the subject, this book fulfils, in part, a long felt need in the field of serologically active bacterial polysaccharides produced by pathogenic bacteria. It would have benefited greatly, however, by a much more critical appraisal and digestion of the literature on the subject. The noticeable monotony of the style makes the reading of the monograph rather burdensome. For workers in the field and persons desiring a ready reference to the various aspects of bacterial polysaccharides discussed, this book can be recommended.

DIAGNOSIS AND TREATMENT OF TUMORS OF THE HEAD AND NECK (NOT INCLUDING THE CENTRAL NERVOUS SYSTEM). By Grant E. Ward, M.D., D.Sc., and James W. Henrick, M.D., M.S., of the Departments of Surgery of the University of Maryland School of Medicine and the Johns Hopkins University School of Medicine, and the Oncology Clinic of the University Hospital and the Tumor Clinic of the Johns Hopkins Hospital. Pp. 832, with 637 illustrations. Price $15. Williams & Wilkins Company, Mount Royal & Guilford Aves., Baltimore 2, 1950.

It is not often that within the compass of a single volume one can obtain a good coverage of the pathological and clinical aspects of neoplasms in a region replete with such diagnostic and surgical difficulties as the head and neck. The authors have approached that ideal in this richly illustrated and, in all, well written textbook.

There is a chapter devoted to the embryology of this region. Essential anatomic details are introduced whenever a specific area of the head and neck is being considered. The pathological appearances of benign growths and cancers are well covered and, as a rule, faithfully illustrated with ample photomicrographs. Some of these, however, particularly those of the skin tumors of the head and neck, are poor or, in a few instances, not at all representative of what they are claimed to represent (for example, figs. 76 *B* and 78 *B*). Clinical manifestations, therapy and general results are given in detail.

For the pathologist, particularly one who is not an active participant in a tumor clinic, this volume contains much that is instructive and informative.

PATHOLOGIC PHYSIOLOGY: MECHANISMS OF DISEASE. Edited by William A. Sodeman, M.D., William Henderson professor of the prevention of semitropical diseases, Tulane University of Louisiana School of Medicine; senior visiting physician, Charity Hospital of Louisiana; consultant in medicine, United States Marine Hospital, New Orleans. Pp. 808, with 146 illustrations and 30 tables. Price $11.50. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia 5; 7 Grape St., Shaftesbury Ave., London W.C. 2, 1950.

This book contains a series of monographs concerned principally with the pathologic physiology of organs or systems involved in human diseases. The authors are clinicians. Their respective fields of interest are presented in such a way that the reader is certain to gain a better understanding of the origin of signs, symptoms and laboratory findings in disease. Chapters on diseases of the blood, the cardiovascular system and the liver are excellent. Other chapters are less thorough and authoritative. There are regrettable omissions, the most important of which is any special consideration of the nervous system. Also, the book may seem superficial and incomplete to those whose interests in the manifestations of disease are at a chemical level. Basic chemistry is lightly treated, or ignored, throughout. Despite these limitations, this book should serve a very useful purpose. If the editor and the authors persist in improving this creditable book, successive editions will become required reading for every third and fourth year medical student and necessary reading for every physician.

NEWER CONCEPTS OF INFLAMMATION. By Valy Menkin, M.D., associate professor of experimental pathology and head of experimental pathology, Agnes Barr Chase Foundation for Cancer Research, Temple University School of Medicine; formerly assistant professor of pathology, Duke University School of Medicine; formerly assistant professor of pathology, Harvard Medical School. Pp. 145, with 67 illustrations. Price $3.50. Charles C Thomas, 301-327 E. Lawrence Ave., Springfield, Ill., 1950.

The author of this small volume is well known for his book, "Dynamics of Inflammation," which was published in 1940. The present volume is based on a series of four lectures which he presented at the Annual Midwest Seminar of Dental Medicine at Maxwellton Braes, Bailey's Harbor, Wis., in 1948. These four lectures amply cover his important investigations on the chemical factors underlying inflammation, which have extended over the past two decades.

This monograph can be read in a few brief hours. For student and investigator the time will be well spent, since it deals with a subject which was regarded as cut and dried following the

observations of Cohnheim and Metchnikoff in the last century but which has been enlivened by the author with new ideas, new names and experimental possibilities.

The quality of the paper, the clarity of the type and the handsome reproductions of Miss Piotti's drawings more than compensate for the number and extent of footnotes, which distract the reader's attention and which seem unnecessary, since many of them could very well have been included in the text.

LEHRBUCH DER ALLGEMEINEN PATHOLOGIE UND DER PATHOLOGISCHEN ANATOMIE AUF GRUND DES RIBBERTSCHEN LEHRBUCHES. By H. Hamperl, O. O. professor, director des Pathologischen Ipstitutes der Universität Marburg. Pp. 788, with 698 illustrations. Price 48 German marks. Springer-Verlag, Berlin, 1950.

This book constitutes the eighteenth and nineteenth edition of a text originally written by Ribbert. It is designed for medical students and consists of clearcut, well written chapters, classified into etiological aspects of pathology, general pathology and systemic pathology. Four hundred and eighty-four pages are devoted to systemic pathology.

The individual chapters are concise, stressing principally the gross appearance of the individual lesions and the various causative factors. The discussion of tumors in general pathology is short, but adequate, and gives the student a good foundation in the modern concept of tumor growth.

It is interesting that a section in the chapter on the heart is labeled "coronary insufficiency" rather than coronary occlusion, indicating a pathophysiological rather than a pathoanatomic approach. In the very short chapter on rheumatic fever called "rheumatism" it is stated that Aschoff nodules are found in the heart, joints, tendons, subcutaneous tissue and galea aponeurotica. It is usually conceded that Aschoff bodies occur only in the heart, while the nodules located in other structures are classified as rheumatic nodules (nonspecific lesions).

There is a short note on diseases of the nose and the antrum, but diseases of the eyes and ears are not discussed. The chapter on the breast is extremely short and rather sketchy. Bones and joints, on the other hand, are adequately treated. An excellent feature of the book is the fact that all terms are given their etymologic explanation. They are literally translated from the original Latin and Greek. Every surname mentioned in the text is explained in a footnote. There are no references in the book; only in a very few instances the reader is referred to more extensive monographs on a particular subject. There are 698 illustrations, photographs and schematic drawings. All the photographs are excellent. The book is of neat appearance, with an easily readable print. The author's German is easily understandable, lengthy complicated sentence structures being avoided. The reviewer feels that this book will occupy an important place in German medical schools but will not be readily accepted in the United States, since it does not treat the material differently, nor does it provide features additional to those contained in most of our standard medical school texts.

SELECTED STUDIES ON ARTERIOSCLEROSIS. By Rudolf Altschul, M.U. Dr., professor of histology, University of Saskatchewan, Saskatoon, Canada. Pp. 182, with 79 illustrations. Price $5.50. Charles C Thomas, 301-327 E. Lawrence Ave., Springfield, Ill., 1950.

This attractively set up monograph deals with human and experimental arteriosclerosis, mostly from the morphological standpoint. It contains the results of the author's experiments in which cholesterol, cholesterol derivatives and cholesterol-like compounds either fresh or heated and dried, were administered to rabbits, guinea pigs, golden hamsters, prairie gophers and white rats. He stresses the importance, in the pathogenesis of arteriosclerosis, of the proliferation of endothelium, by amitosis, dedifferentiation and metaplasia, into various cell forms. This is accompanied with the migration of smooth muscle from the media. The atypical cell forms may change to foam cells. A systematic analysis is made of the tissues and organs involved in experimental cholesterosis.

This monograph serves an important function in reemphasizing the gaps in the morphology of arteriosclerosis.

A Syllabus of Laboratory Examinations in Clinical Diagnosis: Critical Evaluation of Laboratory Procedures in the Study of the Patient. Edited by Thomas Hale Ham, B.S., M.D., assistant professor of medicine, Harvard Medical School; associate director, Thorndike Memorial Laboratory; junior visiting physician, Boston City Hospital. Pp. 496, with numerous illustrations and tables. Price $5. Harvard University Press, Randall Hall, Cambridge, Mass., 1950.

When compared with a textbook, a syllabus designed for a specific course has certain disadvantages. Coverage of material is not so systematic, nor is it complete. But there are certain compensatory advantages, namely, freshness of approach and didactic clarity. Probably few instructors in clinical pathology outside its own institution of origin, will wish to adopt this volume in its entirety, but everyone interested in clinical pathology in both medical schools and hospitals can derive great profit from reference to it. Unfortunately, the book, varityped and reproduced by offset lithography, is fatiguing and physically difficult to read, and is not nearly as satisfactory as a printed volume.

An Atlas of Human Anatomy. By Barry J. Anson, Ph.D., professor of anatomy, Northwestern University Medical School. Pp. 518. Price $11.50. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia 5; 7 Grape St., Shaftesbury Ave., London W.C. 2, 1950.

This new atlas of anatomy is comprised of a collection of superb plates prepared by several outstanding medical illustrators from observations of original dissections. It includes numerous figures indicating variational features of gross anatomy arranged in statistical sequence. The muscular and vascular systems are particularly well covered, and special attention is given to the inguinal region and the female pelvis. The text is abbreviated to little more than extended legends. This rigorous treatment is felt acutely in regard to some of the figures, especially those showing complex anomalous patterns, whose value would be greatly increased by a more generous explanatory comment.

The book will serve as a welcome supplement to the standard anatomic tomes, which are replete with stereotyped and frequently unreliable figures. It will be of considerable practical value to the surgeon, and it should be a useful reference book for the critical pathologist.

A Textbook of X-Ray Diagnosis. By British authors in four volumes. Second edition. Edited by S. Cochrane Shanks, M.D., F.R.C.P., F.F.R., director of the x-ray diagnostic department, University College Hospital, London, and Peter Kerley, M.D., F.R.C.P., F.F.R., D.M.R.E., director of the x-ray department, Westminster Hospital and radiologist, Royal Chest Hospital, London. Volume III. Pp. 830, with 694 illustrations. Price $18. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia 5; 7 Grape St., Shaftesbury Ave., London W.C. 2, 1950.

Advances in Internal Medicine. By William Dock, M.D., Long Island College of Medicine, Brooklyn, and I. Snapper, M.D., Mount Sinai Hospital, New York. Pp. 549, with numerous illustrations. Price $10. Year Book Publishers, Inc., 304 So. Dearborn St., Chicago 4, 1950.

An Index of Tumor Chemotherapy: A Tabulated Compilation of Data from the Literature on Clinical and Experimental Investigations. By Helen M. Dyer, biochemist, National Cancer Institute, National Institutes of Health. Pp. 329. Federal Security Agency, Public Health Service, Bethesda 14, Md., March 1949.

Plasma Proteins: Volume II of the Symposion on Nutrition of the Robert Gould Research Foundation, Inc. Edited by John B. Youmans, M.D., dean and professor of medicine, Vanderbilt University School of Medicine, Nashville, Tenn.; formerly dean and professor of medicine, University of Illinois College of Medicine, Chicago. Pp. 352, with numerous illustrations and tables. Price $6.50. Charles C Thomas, 301-327 E. Lawrence Ave., Springfield, Illinois, 1950.

PRINCIPLES OF GENERAL PSYCHOPATHOLOGY: AN INTERPRETATION OF THE THEORETICAL FOUNDATIONS OF PSYCHOPATHOLOGICAL CONCEPTS. By Siegfried Fischer, M.D., clinical instructor in psychiatry, University of California; formerly professor of psychiatry and neurology, University of Breslau. Pp. 327. Price $4.75. Philosophical Library, Inc., 15 E. 40th St., New York 16, 1950.

THE PHYSICIAN EXAMINES THE BIBLE. By C. Raimer Smith, M.D., D.N.B. Pp. 394. Price $4.25. Philosophical Library, Inc., 15 E. 40th St., New York 16, 1950.

BASIC PRINCIPLES OF CLINICAL ELECTROCARDIOGRAPHY. By Hans H. Hecht, M.D., associate professor of medicine, University of Utah School of Medicine, Salt Lake City. Pp. 88, with 32 illustrations. Price $2. Charles C Thomas, 301-327 E. Lawrence Ave., Springfield, Ill., 1950.

PROGRESS IN BIOPHYSICS AND BIOPHYSICAL CHEMISTRY: Volume I. Edited by J. A. V. Butler, Chester Beatty Research Institute, Royal Cancer Hospital, London, and J. T. Randall, F.R.S., Wheatstone professor of physics in the University of London at King's College. Pp. 279, with numerous illustrations and tables. Price $6.80. Academic Press, Inc., 125 E. 23d St., New York 10, and Butterworth-Springer Ltd., London, 1950.

THE EXTERNAL SECRETION OF THE PANCREAS. By J. Earl Thomas, M.D., department of physiology, Jefferson Medical College of Philadelphia, Philadelphia. Pp. 149, with 22 illustrations and 9 tables. Price $3.50. Charles C Thomas, 301-327 E. Lawrence Ave., Springfield, Ill., 1950.

PHYSIOLOGIE UND PATHOLOGIE DES BILIRUBINSTOFFWECHSELS ALS GRUNDLAGEN DER IKTERUSFORSCHUNG. By Dr. Med. et Phil. Habil. Tr. Baumgärtel. Pp. 271, with 2 illustrations. Price 27 German marks. Georg Thieme, Diemershaldenstrasse 47, Stuttgart-O, 1950.

PHARMACOLOGICAL BASIS OF PENICILLIN THERAPY: By Karl H. Beyer, Ph.D., M.D., director of pharmacological research, Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pa. Pp. 215, with 35 illustrations and 17 tables. Price $4.50. Charles C Thomas, Publisher, 301-327 E. Lawrence Ave., Springfield, Ill., 1950.

LEHRBUCH DER GERICHTLICHEN MEDIZIN. By Albert Ponsold, professor of forensic medicine at the University of Münster, Westphalia, Germany. Pp. 568, with 169 illustrations. Price $11.65. Georg Thieme, Diemershaldenstrasse 47, Stuttgart-O, 1950.

Dr. Ponsold with the assistance of sixteen collaborators has reviewed the various aspects of the problems of forensic medicine. There are included in this text methods for blood grouping, determining Rh factors and examining hair, semen and various other substances of importance in crime detection. The chapter entitled "Gerichtliche Psychiatrie" (Forensic Psychiatry) reviews the more common types of mental derangements as related to medicolegal problems. The section on deaths due to electric current is an excellent addition to this book. The text is well illustrated with charts and photographs and the interpretation of the pathological anatomic changes are brought up to date.

This book is recommended not only to those interested in forensic medicine but to the general pathologist. The latter should be familiar with the technical methods of a medicolegal autopsy. Quite often the hospital pathologist in performing a routine necropsy may encounter anatomic changes which are not in the category of natural causes of death, and the case is referred to the coroner's or medical examiner's office. The pathologist will gain valuable information from Ponsold's book which will supply him with a wider insight into the subject of forensic medicine.

CYTOLOGIC DIAGNOSIS OF LUNG CANCER. By Seymour M. Farber, M.D.; Milton Rosenthal, M.D.; Edwin F. Alston, M.D.; Mortimer A. Benioff, M.D., and Allen K. McGrath, Jr., M.D. From the University of California Medical Service, the Department of Pathology of San Francisco Hospital, and the San Francisco Department of Public Health. Pp. 79, with 60 illustrations. Price $6. Charles C Thomas, Publisher, 301-327 E. Lawrence Ave., Springfield, Ill., 1950.

This monograph on the cytology of the secretions of the respiratory tract follows the general pattern of Papanicolaou and Traut's book on the cytology of the female genital tract. Introductory and historical sections are followed by descriptions of the technic of obtaining and preparing specimens. Incorporated in this chapter are several modifications of the technic recommended by Papanicolaou which will be valuable to workers in this field.

A short discussion of the histological and pathological features of the respiratory tract forms the basis for detailed descriptions of the cells found in secretions from normal and diseased lungs. These will be of particular value to cytologists. Although the 60 colored photomicrographs are among the best one has seen, they do not attain the value of the drawings in Dr. Papanicolaou's book in usefulness in the all-important study of nuclear structure. However, the reproductions in the present book succeed in illustrating many of the critical diagnostic features that are described in the text.

Perhaps the most interesting portion of this work is the section entitled "Technical Advantages for the Study of Cell Details with Wet Smears." In this section the authors discuss the most controversial topic related to cytological work, that of the superiority of this method in some respects over the time-honored method of tissue section examination. This courageous comparison is lucid and thoughtful and will be appreciated by those who have been instrumental in developing cytological methods, as well as edifying to those who still regard it with suspicion.

Of more general interest are the excellent discussion of the problem of pulmonary cancer and, in the conclusion, a critical analysis of the relationship of the cytological method to this problem. In this regard it is significant that the authors agree with Herbert and Clerf, of Philadelphia, and MacDonald, of the Mayo Clinic, that positive cytological findings unsupported by a biopsy showing positive evidence of neoplasm may constitute an indication for thoracotomy in a case of suspected carcinoma of the lung. The usual reservations relative to the experience of the cytologist are strongly stressed, however, and should serve as sufficient warning against abuse of the method.

The authors have succeeded in furnishing guidance to cytologists and have surpassed earlier investigators in presenting sensible and practical solutions to the many problems of application of the cytological method.

RENAL DISEASES. By E. T. Bell, M.D., professor of pathology in the University of Minnesota, Minneapolis. Second edition, thoroughly revised. Pp. 448, with 123 illustrations and 4 plates in color, and 71 tables and charts. Price $8. Lea & Febiger, 600 S. Washington Sq., Philadelphia 6, 1950.

It is a pleasure to read a book written by an authority. This is such a book. Practically every significant statement or point of view of the author is carefully documented by tabulated data, compiled from his personal study of human renal disease. Morphological changes are emphasized in the text and in the many excellent illustrations. But in each chapter the author has always given due consideration to correlating renal structural changes with clinical manifestations and laboratory findings. All may not agree with the author's concepts of the pathological physiology of glomerular and tubular function. In general, his views are conservative and classical. Newer theories are at times neglected or briefly dismissed, especially when they have not been fully treated by analysis of human disease. However, the purpose of this book is not to deal with controversies and minutiae but rather to present the structural basis of renal disease as clearly and forcibly as possible. No one recognizes more clearly than the author those instances in which structural concepts, developed largely by pathologists, fail to yield an adequate explanation for the measured disturbances in renal function. That the author did not give a clearer discussion of these instances is perhaps one minor fault in an otherwise excellent book.

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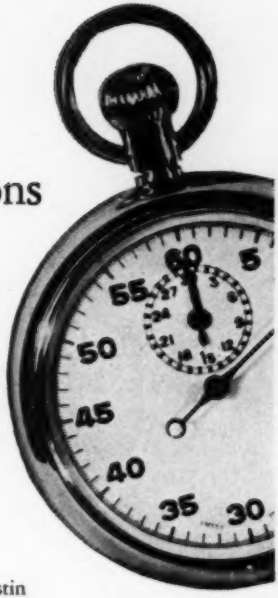

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1. Shapiro, S., Weiner, M., et al.: Am. Heart J. **40**: 766 (Nov.) 1950

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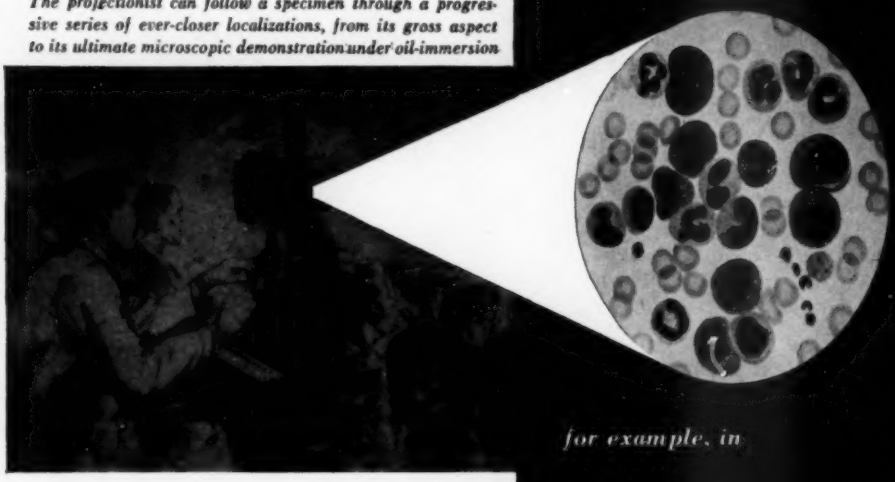

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